THE *cdc2* GENE, ITS CLONING FROM WHEAT, HOMOLOGY WITH YEAST, AND CONTRIBUTION TO DEVELOPMENT IN PLANTS

BY

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STATEMENT

All the research reported in this thesis is original
and my own, except where due acknowledgment is made
and has not been submitted for any other degree

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ABSTRACT

Using RT-PCR followed by nested PCR with degenerate primers based on conserved regions of the *cdc2* gene in the PSTAIR region specific for *cdc2* and other regions specific for related protein kinase, two fragments about 280 bp were obtained from wheat. One was used to probe a wheat cDNA library detecting two genes, that corresponded to these fragments obtained by PCR. The two genes, *cdc2TaA* (882 bp ORF) and *cdc2TaB* (879 bp ORF), showed 85% identity at the amino acid level. Both genes contained characteristics of the *cdc2* gene such as the PSTAIR domain, and had about 60% homology with yeast *cdc2/CDC28* gene, 63% with the human *cdc2* gene and 82%-92% with other plant *cdc2*-like genes.

Complementation tests in budding yeast $cdc28^{ts}$ mutants with both wheat genes under the control of the inducible *GAL1* gene promoter revealed that *cdc2TaA* could promote cell division at restrictive temperature in mutants $cdc28\text{-}13^{ts}$ and $cdc28\text{-}1N^{ts}$ but not $cdc28\text{-}4^{ts}$. Complementation by *cdc2TaA* did not fully restore the wild type rate of cell proliferation analysed in the liquid culture by microscope and flow cytometry. However *cdc2TaB* could not complement mutants $cdc28\text{-}13^{ts}$, $cdc28\text{-}1N^{ts}$ and $cdc28\text{-}4^{ts}$. To test the possibility that *cdc2TaB* might be a close variant of *cdc2*, like the *cdk2* of animals, the encoded amino acid sequences of the wheat *cdc2*-like genes were compared with *cdk2* genes of taxonomically remote animals. The sequence comparison of *cdk2* from *Xenopus*, human and goldfish showed 23 conserved amino acids in *cdk2* that differ from all *cdc2* genes, but in all of these sites wheat *cdc2TaA* and *cdc2TaB* genes encode non-*cdk2* amino acids. The pair of wheat *cdc2*-like genes was also compared with other pairs in alfalfa, soybean and rice. There are 43 sites of amino acids where the two wheat *cdc2*-like genes differed from each other but when compared with a pair of *cdc2*-like genes from alfalfa, of which only one complemented $cdc2^{ts}$ and indicated two genes have different function in the cell cycle, the alfalfa pair shared only 13 of them and in 6 of these sites the amino acid in the non-complementing alfalfa allele MsB differed from that in TaB; thus the 54%

similarity of MsB with TaB at the points where they both shared differences from the other *cdc2*-like gene in each plant, is lower than the overall similarity of 85% between MsB and TaB. The similar result was obtained when the wheat genes compared with the soybean genes. However, the pair of wheat genes is very similar to the rice *cdc2*-like pair, of which again only one complemented *cdc28*^{ts}. Wheat *cdc2TaA* is similar to the complementing rice *cdc2Os1* with 92.5% identity, while *cdc2TaB* is similar to *cdc2Os2* with 91.8% identity. At the 43 places where the two wheat genes differed from each other, the rice pair were also different in 30 of them; and in 22 of these places the amino acids in rice *cdc2Os2* are the same as that in wheat *cdc2TaB*. A structural feature that could account for different capacity for complementation is the identity of amino acid at position 277, which in chick has been reported as a site of phosphorylation. At this site the non-complementing *cdc2*-like alleles of wheat and rice encode Asn that cannot be phosphorylated while the complementing alleles both encode Thr, which can be phosphorylated. These similarities indicate that rice and wheat pairs of *cdc2*-like genes are closely related and the possibility is discussed that they might make specific contributions to the cell cycle or development in both plants.

Northern blots revealed that the mRNA of wheat *cdc2*-like genes was about 1.5 kb and was only abundant in the tissues containing actively dividing, undifferentiated cells, such as root tip, leaf meristem and suspension culture cells; while in the differentiating tissue, such as leaf middle region, and in terminally differentiated tissue, such as leaf tip, *cdc2* mRNA was hardly detectable. This finding was consistent with the change in p34^{cdc2} protein level observed during leaf development, when the protein was quantified with antibody raised against the carboxy terminal peptide revealed by the cloned genes. This antibody was found to be able to detect both *cdc2TaA* and *cdc2TaB* since it could detect both proteins expressed separately in yeast cells. Higher levels of *cdc2* protein relative to others was detected in leaf meristem, while little or none could be detected in the mature leaf blade.

A precise correlation of p34^{cdc2} protein level and kinase activity with development was revealed by p34^{cdc2} level and activity in the small segments of the

7-day-old wheat seedling leaf, which provides a linear gradient of cells progressing through differentiation. Cell division was restricted to the meristem region 0-10 mm from the leaf base where p34^{cdc2} protein was detected at maximum level, but declined to one-fifteenth of this level in the mature cells. The activity of p34^{cdc2}-like H1 histone kinase purified with the p13^{suc1} binding was sharply restricted to the meristem region with the highest level at the first segment (0-4 mm), and declined steeply to one fifth in the second segment (4-8 mm) where mitotic cells also dropped to one fifth. Therefore it is proposed that a fine regulation of p34^{cdc2} kinase activity controls cell division *in vivo*, while cessation of *cdc2* expression and a low level of p34^{cdc2} protein provides a long term regulation for the switch from division to differentiation during plant development.

The decline of p34^{cdc2} protein in mature wheat cells was irreversible. In tissue culture only meristematic cells were able to respond to the presence of the auxin analogue 2,4-D by dividing and forming callus. Auxin treatment could not do more than stimulate partial retention of p34^{cdc2} level and kinase activity. This finding provides a molecular explanation for the recalcitrance of mature monocotyledonous cells in re-entering the cell cycle.

A converse developmental situation observed in pea root led to the study of p34^{cdc2}-like protein involvement in the initiation of cell division. In the region developing into the apical meristem of the radicle, p34^{cdc2}-like protein accumulated linearly during 16-68 h after germination (imbibition) and p34^{cdc2}-like kinase activity increased during the same period and reached a maximum level by 56 h that was twice the activity at 32 h and 44 h and about 10 times the initial activity. These changes of p34^{cdc2}-like protein level and kinase activity coincided with the initiation of cell division and an increase of cell number. In a fully developed pea root, p34^{cdc2}-like protein level and kinase activity was high at root tip and declined in the elongation zone and differentiating zone. The significance of p34^{cdc2}-like genes for cell cycle progress and their likely contribution to plant development is discussed.

GENETIC ABBREVIATIONS

I. For all genes italic characters are used.

Budding yeast genes are given in uppercase, eg, *CDC28*;

Fission yeast genes are given in lowercase, eg, *cdc2*;

Mutant genes are indicated by superscripts of lowercase, eg, $cdc2^{ts}$ signifies temperature sensitive mutation of *cdc2*; $cdc2^{-}$ indicates mutation of *cdc2*. Additionally for budding yeast the mutant form of a gene is signified by giving its name in lowercase, instead of uppercase, eg, *cdc28* is a mutant form of *CDC28*.

II. Gene products are described as $p34^{cdc2}$ (signifying size in kDa of the protein and its identity) or Cdc2 (differing from gene name by the initial capital and non italic form).

III. For a group of proteins, uppercase is used, eg, CDK for cyclin dependent kinase; CKI for cyclin dependent kinase inhibitor.

GENERAL ABBREVIATIONS

ATP	adenosine triphosphate
bp	base pair
BrdU	5'-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CAK	cdk-activating kinase
cdc	cell division cycle
CDK	cyclin dependent kinase
CKI	cyclin dependent kinase inhibitor
conc.	concentration
cpm	count per minute
2,4-D	2, 4-dichlorophenoxyacetic acid
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EDTA	ethylenediamine tetracetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) tetracetic acid
FACS	Fluorescence-activated cell sorter
GA	gibberellic acid

g. f. w. (gfw)	gram fresh weight
GUS	β-glucuronidase
h	hour
IPTG	isopropylthio-β-D-galactoside
kb	kilobase
kDa	kilodalton
MAP kinase	mitogen-activated protein kinase
min	minute
MPF	mitosis promoting factor
MsA	Cdc2MsA (alfalfa Cdc2A)
MsB	Cdc2MsB (alfalfa Cdc2B)
M. W.	molecular weight
NP40	NONIDETP-40, nonionic detergent
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethysulfonyl fluoride
RACE	rapid amplification of cDNA end
RT-PCR	reverse transcription-PCR
SDS	sodium dodecyl sulfate
sec	second
TaA	Cdc2TaA (wheat Cdc2A)
TaB	Cdc2TaB (wheat Cdc2B)
TBS	Tris buffered saline
Tris	*N*-tris (hydroxymethyl)-aminomethane
Tween 20	polyoxyethylenesorbitan monolaurate
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Chapter 1 Introduction

1.1 The cell division cycle

The growth and development of living organisms is dependent on the growth and division of cells. In multicellular organisms there is often also a necessity that division cease prior to specialisation of cell structure and function, termed differentiation. Cell division involves the accurate replication of the genetic information and cellular components of the parent cell and therefore ensures that daughter cells are endowed with the same characteristics as the mother cell. The continuing progression of daughter to mother cell during active proliferation can be called cell division cycle. Unicellular organisms behave as if programed by evolutionary competition to grow and divide as often as they are able. Higher eukaryotes normally start from a single zygotic cell, and progress through a series of cell divisions and cell differentiation to form highly organised adult. Therefore the individual cell of a multicellular organism must divide at a rate consistent with the requirements of the organism as a whole, and also it must integrate division with differentiation in appropriate place and time. The present thesis is concerned with a key cell cycle catalyst and its possible contribution in the switch between division and differentiation in a higher plant.

The cell division cycle in all eukaryotes can be divided into four phases (Howard and Pelc, 1953). The S-phase (DNA synthesis) is fundamental to all cell cycles being the time when chromosomes are replicated and it is complemented by M-phase (Mitosis), when the replicated chromosomes are segregated into two daughter cells. G1-phase is a gap interposed between the previous mitosis and DNA synthesis and G2 refers to a gap after DNA synthesis before mitosis. The succession of G1, S and G2 phases before mitosis can be called interphase. The cell cycle events are highly ordered into dependent pathways in which later events depend upon completion of earlier ones. It has recently been recognised that the cell cycle involves molecular signals that impinge on check

points, which restrain later events and allow their progress only when earlier events have been successfully completed (reviewed by Hartwell and Weinert, 1989; Nurse 1994).

Cells that have ceased division in their normal development can remain healthy for very long periods in the nonproliferating, or quiescent, state that is often called G0. The metabolism in G0 cells is often lower than that of cycling cells (Denhart et al., 1986; Cross et al., 1989), however G0 is the phase in which most cells of a multicellular organism perform their essential specialised structural and metabolic contributions to the organism. G0 phase can therefore be thought of as an ultimate destination of many cell types. G0 cells can sometimes be activated to re-enter the cycle under certain circumstances such as stimulation by growth factors. This thesis compares an irreversible transition to G0 phase that occurs in wheat leaf development with the resumption of division in pea root development.

Great advances in understanding the cell division cycle have been made during the last 10 years. Two key elements have been the exploitation of genetic analysis, which has identified key *cdc* (cell division cycle) genes in yeast (Hartwell et al., 1973; Hartwell et al., 1974; Nurse et al., 1976; Nurse and Thuriaux, 1980; reviewed by Nurse 1990) and biochemical and physiological analysis initially applied to dividing eggs (reviewed by Murray and Kirschner, 1991).

1.2 Finding of a key *cdc* gene *cdc2/CDC28*

Many key cell cycle catalysts of higher eukaryotes have been first discovered in yeasts. The advantages of using yeasts are that the cells are amenable to biochemical and classical genetic analyses, their cell size is easily measured, and presence of both haploid and diploid phases of the life cycle allows the isolation of recessive mutations in haploids and their analysis by complementation in diploids. Genetic investigation of the cell cycle have concentrated on two yeasts: the budding yeast, *Saccharomyces cerevisiae*, and the fission yeast, *Schizosaccharomyces pombe*. A large number of temperature sensitive mutants that arrest the cell cycle at a specific point, so called *cdc* (cell division cycle)

mutants, have been isolated in budding yeast, initially by Hartwell's group (Hartwell et al., 1970; Hartwell, 1971; Culotti and Hartwell, 1971; Hartwell et al., 1973; Hartwell et al., 1974) and fission yeast, initially by Nurse's group (Nurse et al., 1976; Nurse and Thuriaux, 1980).

Genetic analysis in budding yeast has shown that separate genes are required for passage through START in late G1, bud emergence, initiation of DNA synthesis, nuclear division, cytokinesis, and cell separation (Hartwell et al., 1974). For example, a capacity for limited progress towards cytokinesis although nuclear events may be interrupted was recognised because mutants *cdc4*$^-$ and *cdc7*$^-$ caused a failure to initiate DNA replication when shifted from a permissive temperature (23 °C) to a restrictive temperature (37 °C), however these mutants were able to produce a small bud (bud emergence) despite their block in initiation of DNA synthesis. Conversely *cdc24*$^-$ was defective in bud emergence at restrictive temperature, but it still could proceed with DNA synthesis and nuclear division although it could not perform cytokinesis and cell separation. Of particular significance, the *cdc28*$^-$ mutation made cells able to continue growth but not perform any of the early events that mark the START of the cell cycle. The *CDC28* gene is therefore considered a major initiating gene of cell division (Hartwell et al., 1974; Reed 1980).

START is defined as the early event or events in the cell cycle at which the cell becomes committed to DNA synthesis, bud emergence, nuclear division and cell separation (Hartwell et al., 1974). A point analogous to START during the G1 phase of mammalian tissue culture cells, has been referred to as the Restriction point (Pardee, 1989). Cell growth and cell division are usually coordinated at this point; when G1 cells reach a critical size in the presence of appropriate nutrients, they will pass START, initiate DNA replication and prepare for subsequent division, often requiring little additional growth to complete these division processes.

In fission yeast, genetic analysis has revealed a number of genes required for the completion of the cell cycle. For example, *cdc10* is required for DNA synthesis; *cdc1*, *cdc2*, *cdc5*, *cdc6*, *cdc13*, are required for nuclear division (Nurse et al., 1976). Of particular interest to division control in fission yeast is *cdc2*, which is essential at both START and mitosis (Nurse and Thuriaux, 1980; Nurse and Bissett, 1981). The mutant

cdc2 gene allowed a landmark experiment in analysis of the relationship between cell cycle proteins in diverse eukaryotes. The *CDC28* gene was found to complement lack of function *cdc2* in fission yeast although budding and fission yeasts have been separate in evolution for 1,000 million years (Beach et al., 1982). It was later also found the *cdc2* gene with introns removed could conversely complement a *CDC28* mutation in *S. cerevisiae* (Booher and Beach, 1986).

The *CDC28* gene and *cdc2* gene both show homology to known protein kinases (Nasmyth and Reed, 1980; Lorincz and Reed, 1984; Hindley and Phear, 1984). These two proteins share 60% homology of amino acid sequence including the perfectly conserved PSTAIR sequence of 16 amino acids (EGVPSTAIREISLLKE) which is located near the N-terminal and is present in all *cdc2* homologues. Thus, this key cdc protein is conserved in these two yeasts which diverged early during the evolution. Function of the *CDC28* gene at mitosis was first indicated by isolation of an allele that lost only mitotic function (Piggott et al., 1982), and this role was eventually confirmed, by temperature shifts from synchronous cultures, by Reed and Wittenberg (1990).

The first biochemical evidence of a key regulator of the cell cycle came from the microinjection of progesterone treated frog oocyte cytoplasm into untreated oocytes, which responded by resuming meiosis (termed maturation) (Masui and Markert, 1971). This cytoplasmic activity was named as maturation promoting factor, MPF (often later taken to signify mitosis promoting factor). MPF activity was found to be high when the oocyte entered the first meiotic division, disappeared after meiosis 1, and reappeared and maintained at high levels at second meiotic metaphase in the unfertilised egg (Gerhart et al., 1984). This suggested that MPF activity is associated with the metaphase state. MPF has been purified from frog and starfish eggs and revealed as a protein kinase that phosphorylates histone H1 (Lohka et al., 1988; Labbe et al., 1988; Labbe et al., 1989b). Purified MPF from frog eggs contained a 32 kDa protein recognised by antibodies raised against the perfectly conserved PSTAIR region of *cdc2* proteins, it was therefore identified as $p34^{cdc2}$ protein, which has been confirmed by complete sequencing (Gautier et al., 1988). Also present in *Xenopus* MPF was a 45 kDa protein later identified as a *Xenopus* B-type cyclin. The association of the *cdc2* protein with a cyclin type protein

has been recognised as a widely used motif in cell cycle control (reviewed by Pines 1995) as discussed below.

The idea that cell cycle mechanisms are conserved in eukaryotes was extended by the finding of a human *cdc2* homologue, which can complement *S. pombe* $cdc2^{ts}$ mutants and shows 63% identity to fission yeast $p34^{cdc2}$, 58% to budding yeast $p34^{CDC28}$ (Lee and Nurse, 1987). Extending biochemical analysis to somatic cells has shown that human *cdc2* protein is a $p34^{cdc2}$ protein kinase that is catalytically active during mitosis (Draetta et al., 1987; Draetta and Beach, 1988; Draetta and Beach, 1989). *cdc2* homologues have also been found in a wide range of species such as starfish (Arion et al., 1988), chicken (Krek and Nigg, 1989), see urchin (Meijer et al., 1989), *Drosophila* (Lehner and O'Farrell, 1990a), mouse (Th'ng et al., 1990), *Aspergillus* (Osmani et al., 1991) and plants (detailed later in 1.7). It is therefore now widely considered that the mechanism regulating mitosis is universal (Nurse 1990).

1.3 Cyclins associating with $p34^{cdc2}$ as essential subunits

1.3.1 Finding mitotic cyclins

In the early embryonic cell cycle of both frogs (Miake-Lye et al., 1983) and sea urchins (Wagenaar, 1983), protein synthesis was found essential for each mitotic division although net growth of the oocyte was not occurring. Injection of partially purified MPF into embryos arrested by inhibition of protein synthesis induced all of the events of mitosis (Miake-Lye et al., 1983), which suggested that protein synthesis was necessary in the egg for MPF formation or activation. In a study of the individual protein synthesis in the first few cell cycles of sea urchin eggs, Hunt and his colleagues found a protein of 56 kDa which changed in level dramatically during the mitotic cycle accumulating to a maximal level at mitosis and then abruptly disappeared at the end of mitosis, then accumulating again in the next interphase. Because of its cyclic appearance, this protein was named cyclin (Evans et al., 1983) and it is now known that there are several such proteins differing in size and the phase of cell cycle in which they predominate.

Purification of MPFs consistently revealed p34^{cdc2} (Labbe et al., 1988; Gautier et al., 1988; Dunphy et al., 1988; Arion et al., 1988) and another associated 45-60 kDa protein (Evans et al., 1983; Swenson et al., 1986; Lohka et al., 1988; Labbe et al., 1988; Gautier et al., 1988). The p34^{cdc2}-associated protein was identified as a cyclin when cloning of genes for proteins that oscillated in level revealed identity of sequence with the 47 kDa component of purified starfish MPF (Labbe et al., 1989a).

A mitotic cyclin was first identified from clam and named as cyclin A (Swenson et al., 1986). Injection of this cyclin A mRNA into *Xenopus* oocyte could induce oocyte maturation indicating that in the oocyte amounts of cyclin are limiting. Another mitotic cyclin, cyclin B, was isolated by Pines and Hunt (1987) in sea urchin and equivalents were found in *Drosophila* (Lehner and O' Farrell, 1989; 1990b). Thus mitotic cyclins are necessary for mitosis and in oocytes, which have a relatively simple cycle, they are sufficient for initiating mitosis since in *Xenopus* oocyte cell-free extracts destroying endogenous cyclin mRNA caused arrest in interphase and added cyclin mRNA could induce mitosis (Murray and Kirschner, 1989; Minshull et al., 1989).

Evidence has also been derived from cells other than oocytes. In fission yeast the *cdc13* gene, required for entering M-phase, was sequenced and found to encode a cyclin B-like protein, p56^{cdc13} (Hagan et al., 1988; Booher and Beach, 1988; Solomon et al., 1988). Like other cyclins, p56^{cdc13} is destroyed at the end of mitosis and reaccumulated during interphase (Moreno et al., 1989; Booher et al., 1989). Over-expression of *cdc2* suppressed the partial-function mutant *cdc13-117* suggesting that these two gene products interact closely (Booher et al., 1989) and co-immuno-precipitation of p34^{cdc2} and p56^{cdc13} further suggested a physical complex (Booher et al., 1989). More recently, crystallographic investigation of Cdk2, a Cdc2 homologue in higher eukaryotes, and Cdk2-cyclin A complex (DeBondt et al., 1993; Jeffrey et al., 1995) revealed that the binding of cyclin A to Cdk2 induced large conformational changes in Cdk2, which realigned active site residues of Cdk2 in the PSTAIR region and opened the catalytic cleft for substrate binding, so potentially activating the enzyme (subject to appropriate phosphorylation, as will be discussed in 1.5). Sequence analysis of cyclins has revealed a

conserved 100-200 amino acids region called the 'cyclin box' (Nugent et al., 1991) which is involved in binding to p34^{cdc2} (Jeffery et al., 1995).

Individual cyclins show characteristic times of maximum abundance in the cell cycle. For example, although cyclin A can induce oocyte mitosis it is now considered that in somatic cells cyclin A may make a more significant contribution during S phase and early G2 phase, since abundance is maximal at those time and cyclin A immunoprecipitates show greater activity of p34^{cdc2}-like histone kinase at those times (Swemon et al., 1986; Minshull et al., 1990; Girard, 1991). Cyclin B accumulates somewhat later than cyclin A and is more coincident with mitotic MPF (Minshull et al., 1990). Other cyclins accumulate earlier in the cell cycle, in particular the G1 cyclins that will be considered later (1.3.2).

Two factors are important in the control of cyclin abundance; changes in rate of synthesis and breakdown. These elements were first characterised for the mitotic cyclins when Pines and Hunter (1989) showed that cyclin B mRNA was regulated in level during the cell cycle being higher at G2-M Phase, and the protein accumulated steadily during G2 to at least 20 times its level in G1 and was abruptly destroyed at mitosis. Cyclin A accumulates earlier than cyclin B and also it is destroyed earlier than cyclin B (Minshull et al., 1990; Pines and Hunter, 1990).

The proteolytic destruction of cyclins is important in determining their level and is also important in establishing a succession of different cyclins in cycle phases. Since cyclins can direct p34^{cdc2} and like enzymes to different substrates (Peeper et al., 1993) and since different proteins must be phosphorylated in different cycle phases, the species of cyclins that are present in a cell are important in establishing the essential alternation of S phase and M phase. Evidence from budding yeast indicates that proteolytic activity directed to mitotic cyclins remains high after mitosis until the cell next executes START, thus ensuring that mitosis is not attempted until cells have again replicated their genome (Amon et al., 1994; reviewed by Nurse 1994). Conversely, G2 cyclins repress synthesis of G1 cyclins through G2 phase ensuring that DNA is not reduplicated (Amon et al., 1993). There is also a direct negative role of cyclin B, in conjunction with p34^{cdc2}, in repressing repeat replication of DNA, which has been dramatically demonstrated by

mutants of *cdc13*$^{-}$/cyclin B in *S. pombe*. Mutation that at the restrictive temperature destroys cyclin B functional integrity, causes repeated rounds of DNA replication (Hayles et al., 1994). Thus the population of cyclins play a major role in determining ordered cell cycle progression.

The molecular basis of cyclin degradation was first indicated for mitotic cyclins with the demonstration that cyclin B becomes ubiquitinated at time of proteolysis (Glotzer et al., 1991; Luca et al., 1991; Hershko et al., 1991), therefore indicating that it is degraded by one of the major mechanisms of eukaryote protein turnover in which proteases are targeted to ubiquitianted proteins. The significance of ubiquitination in cyclin B proteolysis was confirmed by the finding that removal of a degradation box from the amino terminal of the protein removed the capacity for its ubiquitinisation and caused it to become stable. The significance of cyclin degradation for cell cycle progress was then indicated by failure to complete mitosis in the presence of the stable protein (Murray et al., 1989). Other mitotic cyclins contain the same amino terminal degradation box, but G1 cyclins contain an alternative proteolysis recognition motif comprising a region rich in proline, glutamic acid, serine and threonine (PEST region) at the carboxyl terminal of the molecule (reviewed by Pines 1995). It is now recognised that this class of cyclins also share the ubiquitination mechanism of proteolysis (Deshaies et al., 1995), therefore specificity and temporal control of ubiquitination enzymes to target specific classes of cyclin is an important element in cell cycle progression.

1.3.2 Finding of G1 cyclins; positive regulators

Budding yeast has aided the discovery of non mitotic cyclins that activate p34$^{CDC28/cdc2}$ at the G1 to S phase transition. The first evidence for this class of protein was the isolation of the mutation *whi-1* (Carter and Sudbery, 1980) that resulted in an earlier execution of the START function relative to cell growth and hence smaller cell size (Nash et al., 1988). The molecular basis of this effect was not fully resolved until a screen was conducted for genes that could suppress defects in *CDC28*. The selection could yield proteins that interacted physically with p34^{cdc28} and indeed genes

encoding three cyclin-related proteins (*CLN1*, *CLN2*, *CLN3*) were identified which, when deleted together, resulted in failure to execute the *CDC28/cdc2* dependent START event (Hadwiger et al., 1989b; Richardson et al., 1989). The positive contribution made by these CLNs was underlined by the finding that additional copies of these genes advanced the timing of START (Nash et al., 1988; Cross 1988; Hadwiger et al., 1989b). It is now understood that the *whi-1* mutation is a truncation of *CLN3* cyclin that removes the ubiquitination recognition region (PEST region) resulting in higher levels of the protein earlier in G1 phase. The G1 cyclins are therefore significantly different from the mitotic cyclins in that not only are they essential at a major rate limiting control point, but their level positively regulates the timing of START, whereas higher levels of cyclin B in the physiological range do not advance mitosis (Moreno et al., 1989). It is also possible that *CLN3* has a particularly significant role in balancing growth with division since the *CLN3* is unusual among cyclins in being continuously expressed through the cell cycle thus its amount may signal the total amount of other proteins that has been accumulated. It is plausible that a positive feed back loop can be initiated by adequate levels of Cln3 which involves activation of $p34^{CDC28/cdc2}$ that phosphorylates the Swi4/Swi6 transcription factor with resulting further transcription of *CLN1*, *CLN2* and *CLN3* (Nasmyth and Dirick, 1991; Tyers et al., 1993). The significance of Swi4/Swi6 is underlined by the lethal effect of their deletion and the paramount significance of *CLN* transcription in Swi4/Swi6 function is underlined by the ability of ectopic expression of *CLN* genes to prevent cell death due to lack of Swi4 (Dirick and Nasmyth, 1991; Toone et al., 1995).

The family of cyclins in budding yeast is now known to include not only G1 cyclins (*CLN1*, *CLN2* and *CLN3*) but also *CLB5* and *CLB6* whose abundance at S-G2 indicates equivalence to cyclin A molecules, as well as *CLB1* and *CLB2* which peak at mitosis and are equivalent to cyclin B molecules (Surana et al., 1991; Epstein and Cross, 1992; Schwob and Nasmyth, 1993).

G1 cyclins have been less completely characterised in fission yeast. Using a functional complementation screen, Forsburg and Nurse (1991) isolated a G1-type cyclin *puc1* in fission yeast, which contains a region of similarity to cyclin box regions of *CLN1, CLN2* and *CLN3*. *puc1* can act as a G1 cyclin in *S. cerevisiae* and it has a cyclin-like role

in the fission yeast that is distinct from the role of the B-type mitotic cyclin. However the timing of *puc1* expression is not equivalent to that *CLN1-2* of budding yeast and it remains to be established whether fission yeast has other G1 cyclins.

Thus a model has been established for actively proliferating cells in which cell cycle control of G1/S and G2/M transitions are executed through the alternate association of the *cdc2* gene product, $p34^{cdc2}$, with different types of cyclins; G1 cyclins then A-, B-type cyclins.

1.3.3 Cyclins in mammalian cells

Mammalian cells must have the capacity to abstain from dividing while in a nutrient-rich environment and continuing metabolic activity associated with their differentiated function. They therefore have controls that regulate the transition from active cycling to non-dividing. These controls have no parallel in unicellular organisms, therefore the yeast model has limitations and the evidence from animal cells is largely biochemical.

More cyclins have been discovered in mammalian cells than in yeasts. In human cells, in addition to cyclins A and B, cyclins C, D1, D2, D3 and E were isolated through their ability to complement conditionally defective *CLN* fuction in *S. cerevisiae* (Xiong et al., 1991; Lew et al., 1991; Koff et al., 1991). However overexpression of human cyclins A and B in yeast also complemented *CLN* deficiency, therefore the function of mammalian cyclins C, D, E cannot be judged by the genetic complementation test. One useful form of evidence is the time at which the cyclins are abundant; cyclins C, D and E are normally expressed during the G1 interval in mammalian cells (reviewed by Sherr 1993).

D-type cyclin is more likely involved in the G0-G1 transition following growth factor stimulation. Induction of D type cyclin is highly growth factor dependent, but through the cell cycle of continuously proliferating cells their level oscillates very little and when mitogens are withdrawn, they are rapidly degraded (Matsushime et al., 1991, 1992). Although D-type cyclin synthesis begins at early G1 phase, the associated kinase activity is not exhibited until mid-G1 and increases as cells approach the G1-S boundary

(Matsushime et al., 1994; Meyerson and Harlow, 1994). Cyclin D is found in complex with kinases other than Cdc2 (Cdk4 and Cdk6) and cyclin D-Cdk4 complex phosphorylates the retinoblastoma protein pRb at G1-S, but not histone H1 that is a good substrate for mitotic cyclins-Cdc2 (Matsushime et al., 1992; Dowdy et al., 1993; Ewen et al., 1993). The likely role of cyclin D in responding to growth factor and stimulating growth is further indicated by the stimulation of G1 pregression that is caused by ectopic over expression of cyclin D and also by the ability of over expression of D1 as a result of a chromosome rearrangement to cause parathyroid tumour (Motokura et al., 1991).

A consensus of present evidence concerning mammalian cyclins suggests that: D acts at early G1; E acts at the G1-S boundary; A acts at S phase and G2-M; B acts at the G2-M boundary and M phase (reviewed by Sherr 1993, 1994). Although many cyclins are abundant only in specific phase(s) of the cell cycle their roles in the intact cell are currently difficult to interpret simply from biochemical evidence since the cyclins present at G1 and S phase can bind to more than one protein kinase subunit, termed cyclin dependent kinase (CDK).

1.4 Cdc2, Cdk2 and CDK family

The yeast cell cycle is largely controlled by the activity of a single CDK, p34^{cdc2} or p34^{CDC28}, which complexes sequentially with G1 cyclins (eg. CLNs), S phase cyclins (eg. Clb5,6) and mitotic cyclins (eg. cyclin B). In higher eukaryotes, more p34^{cdc2}-like protein kinases have been found with probable roles that in some cases maybe only peripherally involved in the cell cycle. The first variant p34^{cdc2}-like protein kinase (*Eg1*) was found in *Xenopus* by differential screening of an egg cDNA library to find mRNAs whose rate of translation changes soon after fertilisation (Paris et al., 1991). The Eg1 protein shows high homology to Cdc2 proteins with 65.3% identity to *S. pombe* Cdc2 and 63.6% to human Cdc2. Eg1 also has PSTAIR motif, can be immunoprecipitated by anti PSTAIR antibodies and binds to p13^{suc1}. However, Eg1 cannot complement *S.*

pombe cdc2$^-$ or *S. cerevisiae cdc28*$^-$ mutants. Later, a human Eg1 homologue was independently found by two groups (Elledge and Spottswood, 1991; Ninomiya-Tsuji et al., 1991), and was designated as Cdk2 (cyclin dependent kinase 2). Human Cdk2 protein shares 89% identity to *Xenopus* Eg1 and 65% identity to human Cdc2, it also contains Cdc2 characteristics such as PSTAIR. Human Cdk2 can in some laboratories complement *S. cerevisiae cdc28*$^-$ mutants but not *S. pombe cdc2*$^-$ mutants (Ninomiya-Tsuji et al., 1991). The difficulty of complementing *cdc28*^{ts} (reviewed by Pines and Hunter, 1991) indicates that Cdk2 may not fullfil all of the Cdc2 functions and this view is consistent with the finding that in *Xenopus*, Cdk2 kinase activity is essential for efficient DNA replication but not for mitosis and it is physically associated with cyclin A and cyclin E but not cyclin B (Fang and Newport, 1991). Similarly mammalian Cdk2 can preferentially form complexes with cyclin E and cyclin A rather than cyclin B and is active as histone H1 kinase during late G1 and early S phase (Pines and Hunter, 1990; Tsai et al., 1991; Koff et al., 1991; 1992; Dulic et al., 1992; Pagano et al., 1992; Elledge et al., 1992; Rosenblatt et al., 1992) rather than at mitosis.

Further evidence for specific functions of Cdk2 at S phase rather than mitosis has come from depletion of p33^{cdk2} from *Xenopus* oocyte lysates, which inhibits S phase, whereas depletion of p34^{cdc2} does not effect on DNA replication, but does block entry into mitosis (Fang and Newport, 1991). Conversely human p34^{cdc2} is inactive at G1 phase; becomes complexed with cyclin B and active at G2-M and M phase (Draetta and Beach, 1988; Solomon et al., 1990).

Since Cdk2 kinase acts at G1-S and S phase during the cell cycle it might therefore be considered analogous to *cdc2/CDC28* which at G1-S does not involve change in phosphorylation at Tyr 15 (Hayles and Nurse, 1995). However, there is evidence that p33^{cdk2} activity is regulated by phosphorylation of Tyr15, Thr14 and Thr160 (=Thr161 of human Cdc2), implying that it is post-translationally regulated at G1-S by kinases and phosphatases similar to those that act at G2-M to govern the activity of cyclin B-associated p34^{cdc2} (Gu et al., 1992). Thus presence of Cdk2 in higher eukaryotes may allow the subtle control that is offered by phosphorylation to be available at G1-S phase as well as G2-M phase transition.

Another CDK with a probable direct contribution to the core events of the cell cycle is Cdk4, which is found in complex with cyclin D in mammalian cells (Matauahime et al., 1992; Hanks 1987). Cdk4 shows 44% and 48% identity to human Cdc2 and Cdk2. It lacks the PSTAIRE motif, instead contains PV/ISTVRE, and does not bind to p13^{suc1}. Cdk4-cyclin D phosphorylates pRb protein and a pRb-like protein, p107, but not histone H1 or casein (Matsushime et al., 1992; 1994; Kato et al., 1993; Meyerson and Harlow, 1994). Phosphorylation of pRb by Cdk4 during middle to late G1 phase causes pRb to release E2F, a transcription factor for DNA replication genes, and thereby induces S phase (reviewed by Hinds and Weinberg, 1994). Cdk4 expression is induced when quiescent cells are stimulated with mitogens (Matsushime et al., 1992; Geng and Weinberg, 1993). Thus it is considered that Cdk4-cyclin D complexes have roles controlling G0-G1 and START in animal cells.

Other CDKs have also been detected by PCR amplification of sequences in cDNA that have structural similarity to human Cdc2 (Meyerson et al., 1992) and have been found more remote in sequence from Cdc2 (for example Cdk6 has been called PLSTIRE protein). The function of these more distant variants has not been resolved but it is possible that they are more concerned with the activation of growth at the G0 to G1 phase transition in response to hormone stimulation than with direct catalysis of a core cell cycle event such as DNA replication. This view is supported by the finding that some are tissue specific, consistent with response to a tissue specific growth factor or with contributing a function required by only some cells. For example, Cdk5 is found to be present in brain and neuronal cells and binds to a neuron-specific protein, p35 (Tsai et al., 1994; Lew et al., 1994).

Finally some CDK proteins act on others. The Cdc2 protein is only active at mitosis after phosphorylation of Thr161 (or its equivalent) in the sequence THE common to all Cdc2 proteins. This phosphate is attached by CAK (the Cdc2/Cdk2 activating kinase) that was first isolated as cyclin-dependent-kinase, MO15 (also called Cdk7), previously cloned from a *Xenopus* cDNA library as a p34^{cdc2}-related protein. CAK activates Cdc2 and other Cdks through phosphorylation of Thr161 (Solomon et al., 1992; Fesquet et al., 1993; Poon et al., 1993) and is cyclin dependent. A novel cyclin, cyclin H, has been

found to be associated with MO15 to form CAK (Fisher and Morgan, 1994; Makela et al., 1994). MO15 itself also requires phosphorylation at a homologous position, Thr176, for its activation (Fisher and Morgan, 1994; Labbe et al., 1994), thus it is likely that more CDK proteins remain to be discovered.

It is therefore emerging that in higher eukaryotes there is a *cdc2* gene family, not a single *cdc2/CDC28* gene, and regulation of the cell cycle through Cdc2 and CDKs is more complicated than that in yeasts, although understandable as an elaboration of a highly conserved core mechanism that is still used by yeast.

1.5 Mitotic controls — a network of protein phosphorylation

Genetic studies in fission yeast revealed several genes involved in control of mitotic onset. Both inhibitory and stimulatory functions were identified and suggested that a highly responsive mechanism can restrain mitotic initiation until the successful attainment of completed DNA replication, adequate cell size and growth rate. A mutant *wee1*$^-$ causes cells to enter mitosis earlier, resulting in reduced cell size and indicating that the function lost by mutation is normally inhibitory to mitotic initiation. It has since been determined that *wee1* encodes a protein kinase that phosphorylates Tyr15 of Cdc2 (Russell and Nurse, 1987b; Featherstone and Russell, 1991). Another gene, *mik1*, cooperates with *wee1* in the inhibitory tyrosine phosphorylation of Cdc2 and the two activities explain why loss of *wee1* results in only early mitosis, not catastrophically premature mitosis. Double mutants with *wee1*$^-$ and *mik1*$^-$ give a grossly premature lethal mitotic phenotype, suggesting these genes are important in regulating the mitosis in a negative way (Lundgren et al., 1991). Wee1 activity is modulated by another protein kinase, Nim1 (also called Cdr1), which phosphorylates and inhibits Wee1 thus inducing mitosis (Russell and Nurse 1987a; Coleman et al., 1993; Parker et al., 1993; Wu and Russell, 1993). Nim1 may respond to nutrition thus relating cell cycle progression to cell physiology (Feilotter et al., 1991).

A counteracting activity that balances Wee1/Mik1 inhibition was detected by mutation of the *cdc25* gene. The interaction of the two gene types was first indicated by the epitasis of *cdc25* over *wee1*, there being no *wee1*$^-$ phenotype if *cdc25* was also defective. The relationship is now elegantly accountable at the biochemical level since the product of *cdc25* is a tyrosine phosphatase, which specifically removes the inhibitory tyrosine phosphate on Cdc2 that is generated by Wee1 (Russell and Nurse, 1986; Gautier et al., 1991). The Cdc25 protein is periodically accumulated in the yeast cell cycle but more continuously in animal cells (Moreno et al., 1990; Giard et al., 1992). Over-expression of the activators *cdc25* and *nim1*, or deletion of the inhibitor *wee1*, advances cells into mitosis (Russell and Nurse, 1986, 1987a, b). The activity of Cdc25, although not necessarily the amount of the enzyme protein, rises as cells enter mitosis (Izumi et al., 1992; Kumagai and Dunphy, 1992) and from the foregoing evidence is probably the key initiator. Interestingly, Cdc25 is activated by phosphorylation that can be performed by MPF, so a positive feedback loop is likely (Izumi and Maller, 1993, Hoffman et al., 1993). Some other mitotically activated kinases may also be involved in activating Cdc25 (Kuang et al., 1994). The negative regulators of Cdc25 that depress its activity at anaphase and may participate in mitotic control are believed to be protein phosphatase 1 (PP1) (Walker et al., 1992) and possibly PP2A (Izumi et al., 1992). PP1 has been found to be required for anaphase in *S. pombe* (Ohkura et al., 1989), *S. cerevisiae* (Hisamoto et al., 1994), *Drosophila* (Axton et al., 1990), and *Aspergillus* (Doonan and Morris, 1989).

The regulatory network governing MPF activation at the G2-M transition seems to be universal since Cdc25 homologues have been discovered in budding yeast (defined as MIH1, Russell et al., 1989); in *Drosophila* (defined as *string*, O' Farrell et al., 1989; Edger and O' Farrell, 1989); and in mammalian cells, in which three Cdc25s (Cdc25A, B, C) have been found (Galaktionov and Beach, 1991). The three Cdc25s appear to function at different phases: Cdc25A is required for the G1-S transition (Hoffman et al., 1994; Jinno et al., 1994), whereas Cdc25C is needed for the G2-M transition (Galaktionov and Beach, 1991; Millar et al., 1991); the time of Cdc25B activity is unknown. Furthermore Wee1-like protein kinase have been identified in mammals (Igarashi et al., 1991; McGowan and Russell, 1993), in *Xenopus* (Tang et al., 1993), and

in budding yeast (Booher et al., 1993). However early embryonic cell cycles do not use regulation by tyrosine phosphorylation (Edgar et al., 1994) and in the budding yeast inhibitory phosphorylation is not used to restrain mitosis if DNA replication is not complete as in most cells (Amon et al., 1992; Sorger and Murray, 1992), but rather as a checkpoint for accurate deployment of the cytoskeleton for division (Lew and Reed, 1995).

Some mitotic proteins have not yet been understood in terms of their biochemical contribution. One is p13^{suc1}, originally found by its capacity at raised levels to suppress certain *cdc2*^{ts} mutants (Hayles et al., 1986a, b). p13^{suc1} can physically associate with p34^{cdc2} and passage of cell extracts through a column containing p13^{suc1} depletes p34^{cdc2} (Brizuela et al., 1987). Overexpression of *suc1* leads to a delay of mitotic onset (Hindley et al., 1987; Hayles et al., 1986b), whereas deletion of *suc1* results in cells arresting within mitosis with persistently active p34^{cdc2} kinase (Moreno et al., 1989). However the biochemical function of p13^{suc1} remains unknown although a budding yeast *suc1* homologue (*CKS1*) and human *suc1* homologues (*CKShs1* and *CKShs2*) have been isolated (Hadwiger et al., 1989a; Richardson et al., 1990).

1.6 Contributions from CDK inhibitors (CKIs)

Recently, a number of small proteins have been found to have the property of binding to cyclin-dependent kinases and acting as CDK inhibitors (CKI). They are now recognised as having an important role in the regulation of the cell cycle, especially in G1 phase progression (reviewed by Peter and Herskowitz, 1994b; Hunter and Pines, 1994). Two major roles are recognised; one is to restrain exit from G1 phase in the absence of appropriate signals for non-malignant proliferation, another function is to prevent progress through S phase until DNA damage is repaired or apoptotic cell death is induced.

p21 (also known as Sdi1, Waf1, Cip1, Cap20, or Pic1) is a universal CDK inhibitor, which binds to and inhibits a wide variety of cyclin-CDK complexes, including cyclin D-

Cdk4, cyclin E-Cdk2 and cyclin A-Cdk2 (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993; El-Deiry et al., 1993, 1994). Inhibition of CDK apparently requires the binding of more than one p21 molecule (Zhang et al., 1994). The expression of p21 is directly regulated by the p53, a tumour suppressor protein (El-deiry et al., 1993, 1994). If DNA is damaged in G1 phase, p53 is induced in non-malignant cells and acts as a transcription activator of p21, leading to inhibition of cyclin D-Cdk4 and cyclin E-Cdk2, and arrest of the cell cycle in G1 phase (El-deiry et al., 1993, 1994; Xiong et al., 1993; Dulic et al., 1994). p21 is also found to bind the proliferating cell nuclear antigen (PCNA) which functions in both DNA replication and repair as a subunit of DNA polymerase δ (Waga et al., 1994), however p21 can directly inhibit PCNA-dependent DNA replication, but not interfere with PCNA-dependent DNA repair (Li et al., 1994). In normal cells, most of the cyclin-CDK complexes are found associated with p21 and PCNA, and it is probably significant that this association is absent in most malignant cells (Xiong et al., 1993).

$p27^{Kip1}$ is another CKI with 42% identity in the N-terminus to p21, and also can inhibit the activity of all CDKs; the expression of p27 is constant throughout the cell cycle (Polyak et al., 1994b; Toyoshima and Hunter, 1994). However, p27 and p21 respond to different signals. In normal cells p27 is involved in imposing G1 phase arrest due to transforming growth factor β (TGF-β) and contact inhibition (Polyak et al., 1994a). Although TGF-β treatment does not affect p27 expression (Hannon and Beach, 1994), treatment with TGF-β alters the balance of p27-cyclin-CDK associations and increases the free p27, which eventually binds to cyclin E-Cdk2 complex resulting in a block to S phase.

Additionally some CKI proteins are induced under arresting conditions and a good candidate for TGF-β induced CKI is $p15^{INK4b}$, which is increased to almost 30-fold by TGF-β treatment, and inhibits Cdk4 and Cdk6 (Hannon and Beach, 1994). The binding of $p15^{INK4b}$ to Cdk4/Cdk6 results in dissociation of cyclin D-Cdk4/Cdk6-p27 complex with the result that free p27 can inhibit cyclin E-Cdk2 activity so arresting cells before S phase.

A very similar protein to p15 is p16^{INK4}, which is initially identified as a Cdk4-binding protein in SV40 T antigen-transformed cells (Serrano et al., 1993). p16 inhibits cyclin D-Cdk4/Cdk6 activity by competitive binding to Cdk4/Cdk6 (Hannon and Beach, 1994). The level of p16 is elevated in cells lacking functional pRb, such as in cells transformed by T antigen, suggesting that pRb may suppress p16 expression (Parry et al., 1995). Significantly p16 gene locates on human chromosome 9p21, a region that is often deleted in tumour cell lines (Kamb et al., 1994; Nobori et al., 1994), which leads to the idea that p16 gene is a tumour suppressor gene. There are several other 15-20 KDa proteins found to be related to p16 and p15 (Hannon and Beach, 1994; Chan et al., 1995; Hirai et al., 1995), so that there may be a family of G1 CKIs and tumour suppressors.

Although CKI proteins may be particularly important in multicellular organisms the mechanism of CDK inhibition is also found in budding yeast, where Far1 is a Cdc28-Cln inhibitor induced by the pheromonal mating factor which arrests cells in G1 phase (Peter and Herskowitz, 1994a), and p40^{SIC1} is an inhibitor of Cdc28-Clb (Mendenhall, 1993). Additionally in fission yeast, Rum1 is a p34^{cdc2} kinase inhibitor, that has a role in regulating G1 progression (Moreno and Nurse, 1994). Thus CKIs are probably universal agents in regulation the cell cycle and an integration of negative and positive regulators allows the operation of vital checkpoints at S phase and mitosis.

1.7 *cdc* homologues in plants

Knowledge of the cell cycle regulation in yeasts and animals has led us to believe that the mechanism of the cell cycle regulation is evolutionarily conserved (Nurse 1990). Plant cells that cease dividing *in vivo* due to dormancy, differentiation, or *in vitro* due to nutrient starvation, arrest at principal control points in G1 and G2 (Van't Hof, 1974, 1985); this is in general agreement with the controls operating in other eukaryotic systems but difficulty of manipulating the cellular environment in tissues, or of obtaining suspension cultures in which cells are fully dispersed, have prevented study in plant cells

of how many control points there may be in G1 and G2 and where they are located. Studies in unicellular plant *Chlamydomonas* revealed that the main control point is in late G1 and is coupled to cell size like the START control of yeasts and other eukaryotes (John 1984; 1987). A first indication that the molecular mechanisms of plant cell cycle control might be in common with other eukaryotes was the detection of p34^{cdc2}-like protein in *Chlamydomonas* and higher plants using antibody against PSTAIR-containing protein (John et al., 1989). Recent isolation of a number of *cdc2* gene homologues from plants has more rigorously established the identity of *cdc2* in plants, indicating this element is conserved among yeasts, animals and plants.

cdc2 homologues have been found in maize (Colasanti et al., 1991), alfalfa (Hirt et al., 1991, 1993), *Arabidopsis* (Ferreira et al., 1991; Hirayama et al., 1991), rice (Hashimoto et al., 1992), soybean (Miao et al., 1993), *Antirrhinum* (Fobert et al., 1994), pea (Feiler and Jacobs, 1990; 1991), and *Petunia hybrida* (Bergounionx et al., 1992) (the last two listed were *cdc2*-like gene fragments). All plant *cdc2* homologues encode proteins carrying the complete PSTAIR motif (EGVPSTAIREISLLKE) which is unique to p34^{cdc2} and Cdk2 protein kinase. The amino acid sequences of the plant Cdc2 proteins share over 60% homology with yeast Cdc2/Cdc28 and human Cdc2. p34^{cdc2}-like protein, purified from meristematic tissue of wheat, maize and pea by retention on p13^{suc1}-sepharose, has been shown to possess kinase activity using histone H1 as substrate (John et al., 1991; Colasanti et al., 1991; reviewed in John et al., 1993b). This result also shows that plant p34^{cdc2} homologues bind to p13^{suc1} as do their yeast and animal counterparts. Interestingly, there have almost always been found to be two *cdc2*-like genes in each plant species that has been studied and one or both of them can complement yeast *cdc2*^{ts}/*cdc28*^{ts} mutants (Hirayama et al., 1991; Hirt et al., 1991, 1993; Colasanti et al., 1991; Hashimoto et al., 1992; Miao et al., 1993; Fobert et al., 1994). It is an unresolved question, to which this thesis provides relevant information, whether in plants two *cdc2*-like genes function redundantly or perhaps each in specific cell cycle phases, such as in G1 or G2 as do *cdk2* and *cdc2*. Alternatively the genes might be induced by different developmental signals. In alfalfa, one *cdc2*-like gene, *cdc2MsA*, can complement a fission yeast *cdc2*^{ts} mutant and budding yeast mutation *cdc28-1N*^{ts}, which

is blocked at G2 phase at restrictive temperature; whereas the other, *cdc2MsB*, cannot complement these mutants but conversely it can complement budding yeast *cdc28-4^{ts}*, which blocks predominantly in G1 phase from asynchronous culture at restrictive temperature. In alfalfa it has therefore been suggested that two *cdc2*-like genes act at different control points in the cell cycle (Hirt et al., 1993). However two soybean *cdc2*-like genes are found to be differentially expressed in root and shoot and to have similar capacities for complementing in yeast (Miao et al., 1993). Similarly one rice *cdc2*-like gene is found to respond to GA treatment, while the other is not (Sauter et al., 1995). It is therefore possible that multiple *cdc2*-like genes in plants may respond to different development pathways or growth signals.

Cyclin homologues have been isolated from various plant species, including carrot (Hata et al., 1991), soybean (Hata et al., 1991), *Arabidopsis* (Hemerly et al., 1992; Day and Reddy, 1994; Ferreira et al., 1994a), alfalfa (Hirt et al., 1992), *Antirrhinum* (Fobert et al., 1994), and maize (Renaudin et al., 1994). These cDNA sequences encode cyclins recognisable by the conserved cyclin box, but they are difficult to categorise to type based on sequence similarity elsewhere, however A- or B- type characteristics or mixed A- and B- type features have been reported. Microinjection assays in *Xenopus* oocytes showed that *Arabidopsis*, maize, and soybean cyclins were able to induce oocyte maturation and were therefore reported to be mitotic cyclins, however this assay is not very selective as to species of cyclin (Hata et al., 1991; Hemerly et al., 1992; Renaudin et al., 1994). Expression analysis by RNA gel blot hybridisation using synchronised alfalfa suspension-cultured cells and *in situ* hybridisation with *Antirrhinum* floral meristems and *Arabidopsis* roots have shown that certain cyclin transcripts are restricted to cells in the G2-M transition and some in S-phase, therefore more clearly indicating B-type or A-type function (Hirt et al., 1992; Forbert et al., 1994; Ferreira et al., 1994a).

In early 1995, cyclin D homologues were found in *Arabidopsis* by complementation of a budding yeast strain deficient in G1 cyclins (Soni et al., 1995). Because G1 cyclins are much less conserved than A- and B-type cyclins, which have diverged very little in their central domain, isolation of G1 cyclin homologues in particular cannot be based on sequence similarity. Soni and coworkers isolated a family of *Arabidopsis* cyclin D

homologues (called δ1, δ2, and δ3) which rescued a conditional *CLN*-deficient budding yeast strain. This means of cloning is not in itself a guarantee that complementing cyclin must be of G1 type since other classes of cyclin can complement (reviewed by Sherr 1993). However the *Arabidopsis* cyclins, like human D-type cyclins and other G1 cyclins, lack an N-terminal destruction box but contain potential PEST sequences that may allow proteolysis in G2 phase. Significantly the δ cyclins also contained a LXCXE motif in the N-terminal, which is found only in animal D-type cyclins and in certain viral oncoproteins for binding to the retinoblastoma protein (pRb). Although plant pRb homologues have yet to be identified, the finding of δ cyclins which include the pRb interaction motif indicates that pRb homologues may participate in sequestering transcription factors for cell cycle genes in plant cells as it does in animals. Indeed, PCNA, which was recently shown to be part of cyclin D quaternary complexes, has been isolated from various plants (Suzuka et al., 1991; Hata et al., 1992; Markley et al., 1993). Soni et al.(1995) found that cyclin δ3 is rapidly induced by the plant growth regulator cytokinin and cyclin δ2 is induced by carbon source. This is consistent with the possibility that the δ cyclins act in an analogous manner to mammalian D cyclins.

In alfalfa also, a cyclin homologue *cycMs3* has recently been isolated by selecting for mating type α-pheromone-induced cell cycle arrest suppression in yeast. Its sequence shows homology to A- or B-type cyclins but it may be functionally equivalent to G1 cyclins acting at the G0-G1 transition (Meskiene et al., 1995). *cycMs3* can complement the function of *CLNs* in budding yeast and it has been noted that this is not conclusive of G1 cyclin identity. However the *cycMs3* mRNA is constant in all phases of the cell cycle, and predominantly present in actively dividing tissues. Differentiated G0-arrested cells, induced to re-enter the cell cycle by treatment with plant hormones, accumulated *cycMs3* transcript levels long before the onset of DNA synthesis, while other cyclins, *cycMs1* and *cycMs2* accumulated after *cycMs3* (Meskiene et al., 1995). This has lead to speculation that the B-type cyclins represent a prototype which overlaps the function of G1 and G2 cyclins, because recent findings in yeasts show that some B-type cyclins function at G1-S phase, for example, $Cig1^{+}$ and $Cig2^{+}$ in fission yeast (Connolly and

Beach, 1994; Obara-Ishihara and Okayama, 1994) and *CLB5* and *CLB6* in budding yeast (Schwob and Nasmyth, 1993).

Enzymes responsible for the appropriate phosphorylation of CDKs (CAK, Cdc25, Wee1) have not been found in plants so far. The recently isolated MAP kinase (mitogen-activated-protein kinase) homologues (Duerr et al., 1993; Jonak et al., 1993; Stafstrom et al., 1993; Mizoguchi et al., 1994) and protein phosphatase 1 (Ferreira et al., 1993) may represent some candidates which play a role in activating the CDKs. Use of the inhibitory phosphorylation of p34^{cdc2} on tyrosine to arrest the plant cell cycle has been observed in tobacco cells both in tissue and suspension culture (Zhang et al., 1996). Resumption of mitotic progress due to addition of the cytokinin class of hormone is associated with dephosphorylation of p34^{cdc2}, and furthermore the latent activity of the enzyme from cells arrested at the cytokinin control point can be released *in vitro* by reaction with yeast Cdc25 enzyme (Zhang et al., 1996).

1.8 Features of plant growth and aims of this study

The above survey of cell cycle mechanisms indicates that the basic control of the cell cycle shows similarities between yeasts, animals and plants. However, the multicellular organism must integrate division with development and the developmental strategies of higher plants are different from those of animals, especially with regard to postembryonic development, which continues in plants by means of specialised meristems that contain actively dividing cells and provide new cells for the formation of organs throughout the life of the plant. In contrast, animal organogenesis is accomplished during embryonic development and cell division is very restricted in the adult organism. A distinct characteristic of cell division in plants is that most mature cells, upon stimulation, can de-differentiate and re-enter the cell cycle to develop to a new organ or a new plant; this characteristic is known as totipotency.

The persistence of cell division in plant meristerms underlines the importance of understanding how cells that are actively enlarging, as they are displaced from the

meristem by the flow of new cells, are able to abstain from division and to follow the developmental path to differentiation. Observation of more than tenfold decline in level of p34^{cdc2}-like protein relative to others as cells leave the meristem of wheat leaves (John et al., 1990) or as division ceases in developing carrot cotyledon (Gorst et al., 1991) has lead to the suggestion that the switch from division may be enforced by a decline in level of the key cell cycle catalyst (John et al., 1990, 1993a,b). The decline in level may often simply result from cessation of synthesis while other proteins continue to accumulate (John et al., 1990) and this mechanism is consistent with detection of significant levels of *cdc2* mRNA only in the meristem of the maize leaf (Colasanti et al., 1991) and in root and shoot meristems of *Arabidopsis* (Martinez et al., 1992; Hemerly et al., 1993) and soybean (Miao et al., 1993). However the study of mRNA localisation does not unequivocally indicate where the protein will be abundant nor where it will be catalytically active. The *cdc2* protein in plants can be subject to breakdown following hormone shift, as in pea root elongation zone (John et al., 1993b). The *cdc2* protein may also be present but not enzymically active in plants since it is potentially regulated by availability of cyclin subunits and by tyrosine phosphorylation, as described earlier in this Introduction. The differential expression of cyclin genes in plant cells (Fobert et al., 1994) and the presence of inhibitory phosphate at tyrosine of p34^{cdc2} in late G2 phase (Zhang et al., 1996) suggest that these controls are used. However they may be insufficiently secure, or metabolically too expensive for long term developmental switching, since p34^{cdc2} in the absence of appropriate cyclin can lead to repeat duplicating of DNA (Hayles et al., 1994). Furthermore cyclins may be present to fill roles not connected with the cell cycle (Kaffman et al., 1994), and they are potentially capable of acting in the cycle since their specificity is known to be limited from evidence that all known classes of cyclin can substitute for the CLN1-3 cyclins of yeast (reviewed by Sherr 1993). Similarly, inhibitory phosphorylation may be compromised by phosphatases not normally directed to p34^{cdc2} since division in *S. pombe* can be supported by a phosphatase not normally involved in the cell cycle (Gould et al., 1990). Thus persisting control by long term inhibitory phosphorylation may require the

elaboration of on-going phosphorylation to retain its effectiveness. For these reasons changes in level of p34^{cdc2} can still be considered as significant in plant development.

Prior to the present study, investigations of *cdc2* gene expression have involved measurement of the enzyme protein without accompanying measurement of mRNA (John et al., 1990; Gorst et al., 1991) or measurement directly of mRNA (Martinze et al., 1991) or *cdc2* promoter activity (Hemerly et al., 1993) without measurement of the protein. In the present study measurements have been made of mRNA, enzyme protein and enzyme activity accompanying transition in cell proliferation activity at the leaf and seedling root meristem, to evaluate the possible contribution of transcriptional and post-translational controls.

The molecular cloning of wheat *cdc2*-like genes and their expression are described in Chapter 3, while in Chapter 4, the investigation of functional homology of wheat *cdc2*-like genes with yeast *CDC28* gene is described. Chapter 5 presents the change in level of p34^{cdc2}-like protein and kinase activity during seedling wheat leaf development and *in vitro* culture of excised tissue from the leaf. p34^{cdc2}-like protein level and its kinase activity was also studied during pea root development as described in Chapter 6.

Chapter 2
Materials and Methods

2.1 Materials

Unless otherwise stated general laboratory chemicals were purchased from AJAX Chemicals, Sydney, Australia; BDH Chemicals, Victoria, Australia.

Restriction enzymes and enzymes used in molecular cloning were purchased from Pharmacia; Boehringer Mannheim; Promega; USB; New England Biolabs.

Radioactive isotopes ([^{32}P]ATP, [^{125}I]anti-rabbit-IgG) were purchased from Amersham; Bresatec.

The wheat cDNA library was purchased from CLONTECH (Cat.#: FL1092a).

Wheat seeds (*Triticum aestivum* cv. kite) were kindly supplied by Dr Richard Richards, CSIRO, Division of Plant Industry, Canberra, Australia.

Pea seeds (*Pisum sativum* L. cv. Alaska) were kindly supplied by Prof. Frank Wightman, Carleton University, Canada.

Plasmid pBluescript SK$^+$ was purchased from Stratagene; pYES2 was purchased from Invitrogen.

2.2 Methods

2.2.1 *In vitro* culture of wheat seedling tissue

Wheat seeds were surface sterilised by soaking in 70% ethanol for 40 sec then in 10% sodium hypochlorite for 20 min, then washed with sterilised water 4-5 times. Wheat seedlings were grown from sterilised seeds on the MS agar medium (Murashige and Skoog, 1962, see appendix) for 2 days in dark then 5 days in continuous light at 25° C (Wernicke and Milkovits 1984). Samples were taken from these 7-day-old seedlings.

First leaves of 7-day-old wheat seedlings were cut into 4 mm or 2 mm segments from the base to tip. The segments were placed on MS agar medium supplemented with 2,4-D (2,4-dichlorophenoxyacetic acid) at 0-150 μM and incubated in dark at 25°C for 3-4 weeks. The cultured tissues were then used for assay of p34^{cdc2} protein level and kinase activity.

2.2.2 Determination of cell division activity in plant tissue

7-day-old wheat seedling segments or cultured callus were fixed in acetic acid and ethanol at a ratio of 1:3 for 30-60 min then left in 70% ethanol at 4°C for several weeks. The fixed material was chromatin stained by acetocarmine.

The fixed material was immersed in 1% acetocarmine, which was made by dissolving 1 g carmine in 100 ml boiling 45% (v/v) acetic acid then cooling and filtering. The plant material was stained in acetocarmine for 1-3 h or left in acetocarmine for 2-3 days. The stained material was cut to about 0.1 mm and put on a slide then a drop of a 1:1 mixture of acetocarmine (1%) and 1 N HCl was added on it for 1-3 sec then removed by a piece of filter paper. The material was mounted in 20 μl of a 10:1 mixture of 45% acetic acid and glycerol, covered with coverslip, heated for 1-2 sec over a flame, spreaded by tapping the coverslip gently. The resulting slide was observed under the microscope (Zeiss Axioplan); chromatin was red, cytoplasm was uncoloured and cells in mitosis were readily counted.

2.2.3 Protein extraction and immuno-detection of p34^{cdc2}-like protein

2.2.3.1 Protein extraction from plant tissue

Plant tissue was collected and quickly frozen in liquid nitrogen and ground to a fine powder by a mortar and pestle in liquid nitrogen. Proteins were extracted by mixing of 0.2 g grindate with 200 μl modified ice-cold RIPA buffer in eppendorf tube and vortexing twice for 20 sec then centrifuged at maximum speed (14,000 rpm) in a

microfuge for 5 minutes at 4°C. The supernatant was removed to a new tube and kept on ice for immediate assay.

RIPA buffer was freshly prepared as follow: The stock buffer contained 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 100 mM NaCl, 0.1% Tween-20, 1 mM DTT, 10 μM Pepstatin A, 10 μM Leupeptin, 1 mM Sodium fluoride, 1 mM EGTA, 1 mM Pyrophosphate and 12 μM β-glycerophosphate. This buffer was aliquoted in 1.5 ml and stored frozen at -80°C. Immediately before use sodium orthovanadate was added to a final concentration of 1 mM and PMSF was added to 200 μM.

2.2.3.2 Quantitative assay of protein with Coomassie brilliant blue

Dye reagent was made by dissolving 0.05g Coomassie brilliant blue G250 (Bio-Rad) in 25 ml 95% ethanol, then mixing with 50 ml 85% phosphoric acid, diluting to 500 ml with water. The dye-reagent was filtered and kept at 4°C (Spector, 1978).

A standard curve was determined by assay of ovalbumin at 0-100 μg/ml in 1 ml dye-reagent. Assays and standards contained 100 μl RIPA buffer and were brought to a final volume of 200 μl with water then mixed with 1 ml dye-reagent. The mixture was kept at room temperature for 5 min before measurement of OD at 595 nm.

2.2.3.3 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide solution was prepared as follows:

reagent	12% separating gel (ml)	4% stacking gel (ml)
40% acrylamide and 1.07% bisacrylamide	9.0	1.5
H_2O	5.55	11.4
0.75 M Tris-HCl, pH 8.8	15.0	—
1 M Tris-HCl, pH 6.8	—	1.8
10% SDS	0.3	0.15
TEMED	0.015	0.015
15% ammonium persulphate	0.113	0.0375
total volume (for 1 gel)	30.0	15.0

The Bio-Rad electrophoresis apparatus (protein model) were used and samples containing 50 μg of protein were mixed with an equal volume of SDS sample buffer (S x 2 buffer), which contained 0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 2% mercaptoethanol and 0.002% bromophenol, and heated in boiling water for 3-5 min then loaded on to the gel.

Electrophoresis was carried out at constant current of 25 mA per gel during stacking and 35 mA per gel during separating in the buffer of 2.5 mM Tris-HCl pH 8.3, 19.3 mM glycine and 0.1% SDS. Electrophoresis was stopped when the dye reached the bottom of the gel. The gel apparatus was disassembled and the gel was stained in Coomassie blue (0.2% Coomassie brilliant blue G250 in 40% methanol and 10% acetic acid) or proteins were transferred for Western blotting.

2.2.3.4 Western blotting and immuno-detection in blots

The conventional methods of Western blotting were adopted (Towbin et al., 1979).

After SDS-polyacrylamide electrophoresis, proteins were transferred onto 0.45 micron nitrocellulose membrane (Schlicher & Schule) for 8 hr at 180 mA in transfer buffer which contained 25 mM Tris, 192 mM glycine and 20% methanol, pH 8.3. The nitrocellulose was then immersed in Ponceau stain solution containing 0.4% Ponceau S in 3% trichloroacetic acid for 30 min and washed with water. The fidelity of the transfer was observed and the amount of loaded protein of each sample could be compared by the Ponceau stain. The stained nitrocellulose was routinely photographed at this stage.

The nitrocellulose was transferred to blocking solution, which was 5% milk powder in TBS buffer (10 mM Tris-HCl pH 7.4, 0.15 M NaCl) for at least 1 h with shaking, then washed in TBS with 0.05% Tween-20 for 3 x 5 min with shaking.

The nitrocellulose was then arranged on Nescofilm and overlaid with first antibody. The first antibody used for detection of p34^{cdc2}-like protein was a polyclonal anti-rabbit antibody raised against the PSTAIR region which was diluted at 1:500 in antibody dilution buffer, which contained TBS with 0.05% Tween-20 and 1% bovine serum albumin. After 1-2 h incubation in the first antibody, the nitrocellulose was washed in

TBS 0.05% Tween-20 for 2 x 10 min then TBS 0.2% Tween-20 for another 10 min with shaking.

A second antibody was used as follows:

(a) Anti-rabbit IgG coupled with alkaline phosphatase (Sigma A-8025) was diluted to 1:4000 in antibody dilution buffer, overlaid onto the nitrocellulose and left for 1 h at room temperature. The nitrocellulose was then washed in TBS 0.2% Tween-20 for 2 x 10 min and TBS for 10 min, then rinsed with alkaline phosphatase substrate buffer containing 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl and 50 mM $MgCl_2$ for 5 min. The alkaline phosphatase substrate was covered on the nitrocellulose until the positive bands were clear. The nitrocellulose was immediately removed into water to stop the reaction.

The alkaline phosphatase substrate was prepared freshly by diluting 66 µl of NBT (100 mg nitroblue tetrazolium chloride in 1.3 ml 70% dimethylformamide) and 48 µl of BCIP (100 mg 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt in 2.0 ml dimethylformamide) in 15 ml alkaline phosphatase substrate buffer.

(b) For the purpose of quantitative analysis of detected protein, ^{125}I anti-rabbit-IgG antibody was used as second antibody. The antibody was diluted to 0.5 µCi/ml with antibody dilution buffer and was incubated with nitrocellulose for 2 h at room temperature on a rocker shaker. The nitrocellulose was washed 3 x 10 min in TBS 0.2% Tween-20 with shaking and then dried and exposed for two days to a PhosphorImager screen (Molecular Dynamics). The amount of detected protein could be counted by the PhosphorImager Image Quant Software v 3.0.

2.2.4 Purification of p13 *suc1* protein

2.2.4.1 Overexpression of p13*suc1* protein in *E. coli*

The bacterial strain BL 21, which contains a plasmid in which expression of the *Suc1* DNA open reading frame from *S. pombe* is driven by the T7 promoter, was used to overexpress p13*suc1* following induction of the T7 RNA polymerase with isopropyl-β-D-thiogalactoside (IPTG).

The bacterium was grown in LB medium containing 100 μg/ml of ampicillin overnight and then diluted to 1:100 to a final volume of 2 L. IPTG was added when the OD_{600} of the culture reached 0.4-0.5 to induce expression of p13^{suc1} protein. After 3 h, the cells were harvested by spinning for 10 min at 6000 *g*. The cells were resuspended in cold 20 mM Tris-HCl pH 7.5 supplemented with 1 mM PMSF, and then were lysised by French Press at 0°C.

2.2.4.2 Isolation and purification of p13^{suc1} protein

The p13^{suc1} protein was isolated essentially based on the method described by Labbe et al (1989a). Extracted proteins were dissolved in 50 mM Tris 2 mM EDTA pH 8.0 and solid ammonium sulphate was added to give 30% saturation. The solution was placed on ice for 30 min then spun at 10,000 *g* for 10 min and the supernatant was made 50% saturated with ammonium sulphate. Precipitated proteins were dissolved and extensively dialysed against 50 mM Tris-HCl pH 7.5, 0.05% azide in 3500 kDa cut-off dialyse tubing (Spectrum Medical Industries, Inc.) at 4°C before subject to gel filtration on a G75 Sephadex (Pharmacia) in 50 mM Tris-HCl pH 7.5. Fractions containing p13^{suc1} were identified by electrophoresis and pooled together for Phenyl Sepharose (Pharmacia) chromotography. The column was equilibrated with 0.85 M ammonium sulphate and p13^{suc1} loaded in 0.85 M ammonium sulphate and was eluted by a declining concentration of ammonium sulphate from 0.85 M ammonium sulphate in 50 mM Tris-HCl pH 7.5 to zero of the salt in the same buffer.

Fractions which contained p13^{suc1} protein were identified by OD_{280} and confirmed by gel electrophoresis. The protein was recovered by precipitation with 80% ammonium sulphate at 4°C for 10 min and the pellet was obtained by centrifuging at 10,000 *g* and dissolved in about 5 ml PBS buffer (1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4).

The protein concentration was determined either by Coomassie assay (2.2.3.2), or by measuring absorbance at 260 nm and 280 nm. In later case, the concentration was calculated based on the formula: Protein conc. (mg/ml) = 1.55 A_{280} - 0.76 A_{260}. The purity of p13^{suc1} was checked by electrophoresis on a 15% acrylamide gel.

2.2.5 p34^{cdc2}-like protein kinase assay

The assay of p34^{cdc2}-like protein kinase activity made use of the high efficiency with which p34^{cdc2}-like protein can be bound to p13^{suc1} protein *in vitro* (Brizuela et al., 1987) so that p34^{cdc2}-like protein can be affinity purified by p13^{suc1}-beads and eluted with free p13^{suc1} protein.

2.2.5.1 Preparation of p13^{suc1} beads

The p13^{suc1} beads were prepared by mixing 8 mg of p13^{suc1} with 1 ml of packed CNBr-Sepharose gel (Pharmacia) as follows:

0.5 g freeze dried CNBr-Sepharose was swelled in 100 ml 1 mM HCl for 15 min, washed on a G3 filter unit with 100 ml of 1 mM HCl , then 100 ml of 0.1 M PO_4 buffer, pH 7.5 (2.87 g Na_2HPO_4 and 0.74 g $NaH_2PO_4 \cdot 2H_2O$ in 500 ml), and suspended in an equal volume of 0.1 M PO_4 buffer pH 7.5.

8 mg of p13^{suc1} protein, which was dialysed at 4°C overnight against 2 l 0.1 M PO_4 pH 7.5 was mixed with the CNBr-Sepharose for 2 h at room temperature. Then the coupled gel was washed twice in 10 ml 0.5 M PO_4 buffer pH 7.5 (14.37 g Na_2HPO_4 and 3.70 g $NaH_2PO_4 \cdot 2H_2O$ in 500 ml) and once in 10 ml 1 M NaCl in 50 mM PO_4 buffer pH 7.5. Unreached groups were blocked by suspending the gel in 10 ml 1 M ethanolmine pH 8.0 for 1 h at room temperature then washing the gel in 10 ml 0.1 M Na acetate 0.5 M NaCl pH 4.0, then in 10 ml 0.1 M PO_4 buffer pH 7.5, repeating these washes three times, finally washing in 10 ml PBS. The gel was stored in PBS with 0.01% merthiolate at 4°C.

2.2.5.2 Affinity purification of p34^{cdc2}-like protein (MPF) on p13^{suc1} beads

p34^{cdc2} was affinity purified from p13^{suc1} beads by a modification of published procedures (Labbe et al. 1989a, Blow and Nurse, 1990). The whole procedure of purification of active p34^{cdc2}-like protein was carried out at 4°C to minimise loss of enzyme activity.

Frozen tissue grindate was mixed with NDE buffer (150 µl per 0.05 g), vortexed 3 x 20 sec, spun at full speed in a microfuge at 4°C for 5 min. The NDE (No Detergent salt Extraction buffer) was freshly made and contained 20 mM Hepes, 100 mM NaCl, 15 mM DTT, 20 mM EGTA, 50 mM NaF, 15 mM $MgCl_2$, 0.2 mM ammonium molydate, 30 mM p-nitrophenyl phosphate diNa, 1 mM Na orthoVanadate, 80 mM β-glycerophosphate, 3 µg/ml leupeptin, 0.5 mM PMSF, pH 7.4. These components were inhibitory to phosphatases and proteases.

Soluble extract was pre-cleared by adding to 130 µl of extract 40 µl Sepharose 4B which had been washed in NDE. The mixture was rotated for 1 h at 4°C, then spun at full speed for 5 min, the agar was discarded.

To 130 µl of the cleared extract 40 µl p13^{suc1} beads in NDE were added and the mixture was rotated for 1 h at 4°C to allow binding of p34^{cdc2}-like protein kinase, then centrifuged at half speed in a microfuge at 4°C for 2 min, to discard unbound proteins in the supernatant.

The p34^{cdc2}-p13^{suc1} beads were washed twice with 400µl HDW buffer, which consisted of 2 mM EDTA, 150 mM NaCl, 1% NP40, 5 µg/ml leupeptin, 0.1 mM Na ortho Vanadate, 50 mM NaF and 10 mM Na-PO_4 buffer, pH 7.0. Then the beads were washed once in PBS-Mg-DTT buffer, which consisted of 10 mM $MgCl_2$ and 1 mM DTT in PBS buffer.

Finally p34^{cdc2}-like protein kinase was eluted with free p13^{suc1} by adding 50 µl of 0.5 mg/ml p13^{suc1} solution to the beads and rotating on a wheel for 30 min, spinning down the beads at full speed for 5 min . The supernatant was removed and kept on ice until assay.

2.2.5.3 p34^{cdc2} Histone H1 kinase assay

Assay of p34^{cdc2}-like protein kinase was based on the phosphorylation of H1 histone in the presence of [γ-^{32}P]ATP. The amount of [^{32}P] transferred to H1 histone was measured.

20 µl of enzyme prepared as described in 2.2.5.2 was added to 30 µl of reaction mixture contained 25 mM β-glycerophosphate pH 7.3, 10 mM EGTA, 10 mM $MgCl_2$, 1

mM DTT, 25 mM HEPES pH 7.3, 0.1 mg/ml H1 histone (Boehringer), 2 μM ATP and 0.25 μCi [^{32}P]ATP. The reaction continued at 30°C for 5 min then was terminated by transfer to ice.

20 μl of the reaction was pipetted on P81 phosphocellulose paper (10 x 20 mm) and the paper was immediately put in 75 mM phosphoric acid to remove free ATP. The P81 papers were then washed in 75 mM phosphoric acid 5 x 3 min and immersed in 1.5 ml scintillant cocktail. The bound radioactivity on the paper was counted in Liquid Scintillation Systems LS 3801 (Beckman).

The remaining 30 μl of reaction mixture was mixed with an equal volume of SDS sample buffer (S x 2) and boiled for 3 min, then separated on 12% acrylamide gel and stained with fresh Coomassie solution (0.1% Coomassie blue G250, 45% methanol and 10% acetic acid) for 4 h, then destained with a solution of 45% methanol, 10% acetic acid for 1 h followed by 5% methanol and 7.5% acetic acid.

The gel was dried using the Bio-Rad gel drier model 583, then exposed on the PhosphorImager cassette for 12-24 h. The image of phosphorylated histone H1 was obtained by PhosphorImager analysis.

2.2.6 Genomic DNA extraction from plant tissue

Wheat genomic DNA was extracted by the CTAB (Cetyltrimethyl-ammonium bromide) method with a modification of published procedures (Murray and Thompson, 1980).

To 4-5 g of frozen tissue power, 25 ml CTAB buffer (140 mM Sorbitol, 220 mM Tris-HCl pH 8.0, 22 mM EDTA, 800 mM NaCl, 1% Sarkosyl and 0.8% CTAB) was added, then the mixture was incubated at 65°C for 20 min with occasional vigorous shaking. 10 ml of chloroform was added and left at room temperature for 20 min with occasional shaking.

The aqueous phase was recovered by centrifugation at 1088 *g* for 5 min, and transferred to a fresh tube, then an equal volume of isopropanol was added, mixed and

placed on ice for 10 min. The pellet was collected by centrifugation at 1088 *g* for 5 min then dissolved in 4 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

RNA was precipitated by adding equal volume of 4 M LiCl, incubating on ice for 20 min or more, then centrifuging at 1088 *g* for 20 min. The resulted supernatant was transferred to a fresh tube, then mixed with 2.5 volumes of ethanol on ice for 20 min or more to precipitate the DNA.

DNA was recovered by centrifuging at 1088 *g* for 5 min, dissolved in 1-2 ml TE by gentle shaking overnight at 4°C.

Any trace of RNA was removed by adding 1 µl of 10 mg/ml RNase A (DNase free) and incubating at 37°C for 1 h. Proteins were then removed by adding 1/10 volume of 3M NaAc pH 5.3, dividing into several effendorf tubes and extracting with 0.5 ml phenol, then phenol/chloroform, then chloroform.

The extracted aqueous phase was removed to a clean tube and mixed with 2.5 volumes of ethanol on ice for 5 min. DNA was pelleted at full speed of a microfuge for 5 min, washed with 70% ethanol, partially dried then dissolved in an appropriate volume of TE buffer.

The concentration of the DNA was determined by measuring OD_{260} and taking 50 µg/ml DNA solution to have an absorbance of 1 at 260 nm, and a A_{260}/A_{280} absorbance ratio of 1.8 or more to indicate a pure DNA solution (Sambrook et al., 1989).

2.2.7 Extraction of RNA from plant tissue

Tubes, tips and solutions used in RNA work were made RNase-free by keeping in 0.1% (v/v) DEPC overnight then autoclaving for 20 min at 120°C.

2.2.7.1 Extraction of total RNA

A LiCl method was used for extraction of total RNA, Based on that of Vries et al. (1988).

To 1 g frozen wheat seedling powder was added 4 ml of 1:1 mixture of RNA extraction buffer and phenol with hydroxyquinoline, which was pre-heated to 90°C, and

the mixture was shaken for 5 min at room temperature. RNA extraction buffer consisted of 100 mM LiCl, 1% SDS, 100 mM Tris-NaOH, pH 9.0, 10 mM EDTA. Phenol with hydroxyquinoline was 0.1 g hydroxyquinoline added to 100 ml distilled phenol (GIBCO BRL Tris-HCl buffer saturated, pH 7.4) kept at 4°C.

To the phenol-powder mixture 2 ml chloroform was added and shaking was continued for 15-30 min at room temperature.

The aqueous phase was recovered by centrifuging the mixture at 20000 *g* for 30 min at 25°C and removing the upper phase to a clean tube, 2 ml chloroform was added and shaken for 15 min. The aqueous phase was again removed by centrifuging the mixture at 12000 *g* at 25°C, and aliquoted to effendorf tubes. To each sample an 1/3 volume of 8 M LiCl was added, mixed well and RNA allowed to precipitate for 16-48 h at 4°C.

RNA was recovered by centrifuging at full speed in a microfuge at 4°C. The RNA pellet was washed once with 2 M LiCl at 4°C and twice with 80% ethanol, dried and dissolved in double-distilled water and stored at -20°C.

The concentration of RNA was determined by measuring OD_{260}; taking a 40 µg /ml RNA solution to have an absorbance of 1 at 260 nm (Sambrook et al., 1989).

2.2.7.2 Extraction of mRNA

Poly(A)$^+$ RNA was recovered using biotinylated-oligo(dT) from total RNA. 0.1-1.0 mg of total RNA in 500 µl RNase-free water was heated at 65°C in a heating block for 10 min, then 3 µl of Biotinylated-Oligo(dT) probe (Promega PolyATract, reagent) and 13 µl of 20 x SSC (3 M NaCl and 0.3 M Na_3 citrate) were added. After gentle mixing, the probe was allowed to anneal at room temperature for about 10 min.

Poly(A)$^+$ RNA was then recovered by mixing with 100 µl Streptavidin Paramagnetic Particles (SA-PMPs), previously washed three times in 0.5 x SSC. After 10 min at room temperature the SA-PMPs along with biotin(dT)-mRNA hybrids were recovered using the Magnetic Stand.

The particles were washed four times with 300 µl 0.1 x SSC by gently flicking the tube and resedimenting the particles by the Magnetic stand. After the final wash, as

much of the aqueous phase as possible was discarded without disturbing the SA-PMP particles.

mRNA was eluted by resuspending the SA-PMP pellet in 100 µl of RNase-free water which breaks the bonding of oligo(dT) with poly(A) due to low salt. The beads were discarded and the eluted mRNA transferred to a sterile, RNase-free tube. This water elution step was repeated once.

The concentration and purity of the eluted mRNA was determined by spectrophotometry. A Pharmacia LKB Ultrospec III was used to measure a 50 µl sample and the eluted mRNA gave an A_{260}/A_{280} absorbance ratio of 1.9-2.0, indicating acceptable purity.

2.2.8 DNA amplification by Polymerase Chain Reaction

The polymerase chain reaction (PCR) can be used to amplify a fragment of DNA that lies between two regions of known sequence. The DNA is first denatured to single-strands at high temperature (94-96°C) in a reaction mixture that contains two oligonucleotides (primers) complimentary to regions of the template DNA that are about 50-2000 base pairs apart, and four deoxynucleotide triphosphates (dATP, dGTP, dCTP, dTTP) as well as a thermostable DNA polymerase. The temperature is then cooled down to allow two oligonucleotide primers anneal to the target single-stranded DNA and then extended with DNA polymerase at the polymerisation temperature. The cycle of denaturation, annealing and DNA synthesis is repeated many times, resulting an exponentially increased product that is a segment of double-stranded DNA whose length is defined by the distance between the primers, when bound to the template DNA.

2.2.8.1 Primer design and DNA amplification

Primers used in PCR were generally designed to be 18-24 bases with similar G + C content, and with a flanking restriction sequence at their 5' termini (eg. *Eco*RI or *Bam*HI sites) for easier cloning of the amplified products into sequencing, or other, vector. To clone wheat *cdc2*-like genes, degenerate oligonucleotides were designed to complement

regions of highly conserved protein sequence in p34^{cdc2}. The sequences of primers are described in Chapter 3 (3.2.1).

Primers were synthesised on an Applied Biosystems 380 B DNA synthesiser in JCSMR (John Curtin School of Medical Research, ANU), obtained in a concentrated ammonia solution. The oligonucleotides were recovered by mixing 130 µl of this solution with 1.3 ml n-butanol, vortexing for 30 sec, and spining for 2 min in a microfuge. The pellet was dried and dissolved in 40 µl water or TE. The concentration of the primer was determined by measuring OD_{260}, taking 33 µg/ml of oligonucleotide solution to have an absorbance of 1 at 260 nm. The concentration was also calculated in pmol/µl according to the formula (Sambrook et al., 1989): $C = OD_{260}/\varepsilon$.

Here C is µmol/ml concentration, ε is the extinction coefficient of the oligonucleotide derived from the extinction coefficient of the bases multiplied by the number of times each occurred in the oligonucleotide (ε = a dATP + b dGTP + c dCTP + d dTTP. dATP = 15.4 ml/µmol, dGTP = 11.7 ml/µmol, dCTP = 7.3 ml/µmol, dTTP = 8.8 ml/µmol, and units indicate the volume in which 1 pmol is dissolved to give an OD_{260} = 1.0).

For degenerate oligonucleotides, more approximate estimates were calculated as follows:

1 OD_{260} indicates approximately 33 µg/ml and C (µmol/ml) ≅ OD_{260}/10 x length of oligonucleotide.

PCR amplification of DNA segments was normally performed in a 50 µl reaction mixture containing PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 0.25 mM each of dNTPs, 50-100 pmol of each 3' primer and 5' primer, template DNA (0.5-1 µg for genomic DNA, less for cDNA and plasmid DNA), and 2.5 units of Taq polymerase (Boehringer or Pharmacia). The mixture was overlayed with mineral oil to prevent evaporation, placed in a DNA Thermal Cycler (Perkin-Elmer Cetus Model 480) that allows for controlled variation in time and temperature parameters. The PCR temperature cycle usually comprised: 94°C 5 min for initial denaturation; then 94°C 1 min, 55°C 2 min, 72°C 1-3 min depending on the length of amplified DNA, repeated for 30 cycles. The parameters changed in some cases and these are noted where relevant.

The resulting products of PCR were analysed by agarose gel electrophoresis. Usually 5-10 µl of the reaction mixture was separated on 1%-2% agarose gel in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) or TBE (90 mM Tris-borate, 2 mM EDTA, pH 8.0) buffer, stained with 0.5 µg/ml ethidium bromide and photographed on Polaroid film 557 on a UV illumination.

2.2.8.2 Reverse-transcriptase PCR and nested PCR

DNA can be amplified from a single-stranded RNA template by combining a standard PCR protocol with an initial incubation with a reverse-transcriptase enzyme (Sambrook et al., 1989). This strategy was useful for isolation of genes from a big genome.

The primer used in the initial reverse-transcriptase reaction was either oligo(dT) which anneals to the poly(A) tail of mRNA or a synthetic oligonucleotide complementary to a sequence present in mRNA of the gene of interest.

First strand cDNA was synthesised in a 20 µl mixture containing 1 µg total RNA, 1 x PCR buffer, 50 pmol oligo$(dT)_{15}$ (Boehringer) or a 3' primer, 1 mM each of dNTPs, 40 U RNase inhibitor (from human placenta, Boehringer), 100 U murine reverse transcriptase (M-MuLV, Boehringer). The mixture was incubated at 37°C for 30 min, then heated at 94°C to inactivate the reverse transcriptase.

The reaction was then quick-chilled on ice and the mixture was supplemented with a pair of primers (50 pmol each) or 50 pmol of second primer (5' primer) if a 3' primer was used in the reverse transcriptase reaction. The mixture was then brought to 50 µl with PCR buffer, water and 2.5 U of Taq polymerase, overlaid with mineral oil, thermocycled at 94°C 1 min, 55°C 2 min, 72°C 3 min for 30 cycles.

To improve the sensitivity and specificity of PCR amplification nested PCR was applied. The first PCR contained an external pair of primers and 1/25 to 1/50 of this reaction was used as a template DNA source for a second PCR reaction in which two nested primers that were internal to the first pair of primers were used. 25 cycles were performed for nested PCR.

2.2.8.3 Rapid Amplification of cDNA Ends (RACE)

Rapid Amplification of cDNA Ends (RACE) was used as a PCR technique by which the previously unidentified 3' and 5' ends of a cDNA could be amplified starting with the knowledge of a small stretch of sequence within the gene that was obtained in an initial PCR product (Frohman et al., 1988). In this research, RACE was used to amplify the 3' end of wheat *cdc2*-like genes. The strategy is illustrated in Fig. 2.1.

For RACE PCR the first strand cDNA was synthesised as in RT-PCR (2.2.8.2) by using oligo$(dT)_{15}$ as a primer. After heat inactivation of the reverse transcriptase, 1/25 of this reaction was used as template of the RACE PCR, which was carried out in PCR buffer with 0.25 mM each dNTPs, 50 pmol dT_{17}-RI that was oligo$(dT)_{17}$ flanked by a *Eco*RI sequence at 5' end, and 50 pmol 5' primer corresponding to the gene of interest, 2.5 U Taq polymerase. The first reaction cycle was: 94°C 1 min, 55°C 1.5 min, 72°C 2 min; the following 5 cycles were 94°C 1 min, 37°C 1.5 min, 72°C 2 min, the 37°C annealing temperature allowed oligo(dT) to hybridise well with the template. Then 20 cycles of 94°C 1 min, 55°C 1.5 min, 72°C 2 min were given and a final extension time of 7 min was allowed.

Nested PCR was applied by using 1/50 of RACE PCR as a template, then dT17-RI and an internal 5' primer were used as primers, which constituted single nested PCR. 30 cycles were performed at 94°C 1 min, 57°C 1.5 min, 72°C 2 min.

2.2.9 DNA cloning in plasmid vectors

2.2.9.1 Purification of DNA

To clone PCR products they were separated on 1-2% agarose gel in TAE or TBE buffer then stained with 0.5 µg/ml ethidium bromide and the selected bands were cut out. The DNA was extracted and purified with "Prep-A-Gene" (Bio-Rad) or "QIAEX gel extraction kit" (QIAGEN) according to the manufacturer's instructions.

The basic principle of these kits is that the DNA can be bound to silica gel particles in the presence of high salt. Non-nucleic acid impurities such as agarose, proteins, salts and ethidium bromide are removed from the sample by several washes in high salt buffer

and ethanol-containing buffer. The DNA is then eluted in low salt solution such as TE buffer at pH 8.0. The resulting DNA is usually suitable for ligation, labelling, or sequencing reactions.

In practise the agarose piece containing a desired DNA fragment was incubated in three volumes of the solubilization buffer at 50°C for 10 min to disperse the gel, than 5-10 µl of silica gel particles was added and mixed well. The mixture was incubated at 50° C for 10 min to allow the DNA to bind and then spun down. The pellet was washed twice with washing buffer I and twice with washing buffer II, then dried in air. DNA was finally eluted with 10-20 µl of TE buffer.

2.2.9.2 Restriction enzyme digestion and DNA ligation

Both DNA fragment and vector DNA was digested at 37°C for 2 h in buffer supplied by manufacturers as appropriate for the particular restriction enzymes. The enzymes always accounted for less than 0.1 volume of the reaction to avoid concentrations of glycerol in the final reaction that might affect the digestion. The reaction was stopped by heating at 65-85°C to inactive the enzymes.

If a single restriction enzyme that created the same sticky end at both of the linearized vector ends was used; or if the enzyme (eg. *Sma*I) resulted in blunt ends, then dephosphorylation of linearized vector DNA was applied to prevent vector self-ligation. Shrimp Alkaline phosphatase (USB) was used at 1 U per reaction in buffer supplied by the manufacturer for 30 min at 37°C, then heated at 70°C for 10 min to inactive the phosphatase.

After digestion and dephosphorylation, the DNA fragment and vector DNA was separated by agarose gel electrophoresis then purified with an extraction kit as described in 2.2.9.1.

The concentration of vector DNA and foreign DNA was estimated in agarose gel by comparing with a parallel loaded molecular weight marker of known concentration. For ligation, vector DNA and insert DNA was mixed at approximately 1:1.5-2 ratio of molar concentration and incubated in 10-20 µl ligation mixture containing of 5-10 U of T4 DNA ligase (New England Biolabs) and 1x ligation buffer which consisted of 50 mM

Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/ml bovine serum albumin. The ligation reaction proceeded at room temperature (about 22°C) for 1-2 h then at 16°C overnight. The resulting DNA was ready for transformation into competent cells.

2.2.9.3 Preparation of competent cells and transformation

E. coli strains DH5α or XL1-blue were used as host stains and were grown in 2.5 ml SOB medium (see appendix) overnight at 37°C with vigorous shaking. Then the culture was diluted to 1/100 in fresh SOB medium for 2-2.5 h further growing until the OD_{600} reached 0.4-0.6. The cells were then ready for inducing competence by chemical treatment according to Hanahan's method (Sambrook et al., 1989).

Briefly, 20 ml of the culture was divided into 2 chilled Falcon tubes, stood in ice for 10 min, and spun at 5000 g for 5 min. The pellet was retained and resuspended in 5 ml of ice-cold transformation buffer (TFB) which contained 10 mM MES pH 6.3, 100 mM RbCl, 45 mM $MnCl_2$, 3 mM $[Co(NH_3)_6]Cl_3$, and stood in ice for 10 min. Cells were spun as before and resuspended in 200 µl of TFB then cells from two tubes were combined and supplemented with 14 µl of ice-cold DnD (1.53 g DTT, 100 µl 1 M KAc, 9 ml DMSO in a total volume of 10 ml with water). After 15 min on ice another 14 µl of DnD added to the cells which were then kept on ice for a further 15 min. Cells were then ready for transformation.

Competent cells were prepared freshly in this way for every transformation. This method was found 1000 times better than $CaCl_2$ method in terms of transformation efficiency.

For transformation half of the ligation mixture was usually incubated with 40-50 µl of fresh competent cells and kept on ice for 30 min or more. The cells/DNA mixture was then heat shocked for 90 sec at 42°C, followed by recovery of cells on ice for 5 min. Then cells were grown at 37°C for 0.5-1 h in 400 µl SOB medium with slow shaking. Cells were then plated on selective media, usually containing 50 µg/ml of ampicillin, supplemented with 5 µl of 200 mg/ml IPTG and 40 µl of 20 mg/ml X-gal per plate for

selecting white colonies which harbouring plasmid with insert DNA while the colonies containing unmodified plasmid remained blue.

2.2.9.4 Analysis of positive colonies by testing for presence of insert

Single white colony was picked and grown in 2.5 ml LB medium (see appendix) with 50 µg/ml of ampicillin. The overnight culture was used for isolation of plasmid DNA by alkaline lysis according to Sambrook et al., (1989):

1.3 ml culture was spun and the supernatant was removed by aspiration. The pellet was resuspend in 100 µl of ice-cooled Solution I consisting of 50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, then 150 µl of Solution II was added and mixed by inversion. Solution II was 0.2 N NaOH, 1% SDS, freshly mixed from concentrated NaOH and SDS solution. The mixture was kept on ice for 5 min, then 200 µl of ice-cold solution III containing 3 M potassium and 5 M acetate (60 ml of 5 M KAc, 11.5 ml of glacial acetic in a total volume of 100 ml) was added and kept on ice for another 5 min. The cell debris was then spun down and the supernatant was carefully removed to a clean tube, then 2.5 volumes of ethanol were added to precipitate the plasmid DNA. The DNA was spun down and washed with 70% ethanol, dried, and dissolved in 50 µl of TE. 5 µl of the DNA solution was used in restriction enzyme digestion supplemented with 1 µl of 1 mg/ml RNase. The digested DNA was analysed on an agarose gel to inspect presence of an insert band.

2.2.10 DNA sequencing

Sequencing of cloned DNA was performed by the dideoxy chain termination method (Sanger et al., 1977) adapted for use in PCR. Templates were double or single stranded DNA, and reactions were performed according to the manufacturer's directions. The success or failure of sequencing reaction depended on the quality of DNA template.

2.2.10.1 Preparation of template DNA for sequencing

A modified mini alkaline-lysis/PEG precipitation procedure for plasmid DNA was used, based on the protocol of the "PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing kit" (Applied Biosystems).

The major differences from the routine alkaline-lysis method (2.2.9.4) were:

(1) Instead of LB medium, Terrific Broth medium (see appendix) was used to grow bacteria and adequate aeration was provided by fast shaking. These factors resulted in a high yield of plasmid DNA, normally 15-20 µg DNA per 1.3 ml culture.

(2) After alkaline lysis, the supernatant was treated with RNase A (DNase-free) then extracted twice with chloroform to remove RNA and proteins.

(3) A key feature of the improved protocol was the inclusion of a PEG precipitation step, which yielded high-quality super-coiled plasmid DNA that was relatively free of contaminating chromosomal DNA and RNA.

Briefly, the cell pellet was resuspended in 200 µl Solution I, then mixed with 300 µl Solution II, and then 300 µl Solution III. After removed the cellular debris by spinning, the supernatant was supplemented with RNase A to a final concentration of 20 µg/ml and incubated at 37°C for 20 min, then extracted with 400 µl of chloroform twice. The resulting aqueous phase received 1 volume of 100% isopropanol and was spun immediately. The DNA pellet was washed with 70% ethanol, dried, dissolved in 32 µl of H_2O. The plasmid DNA was then reprecipitated by first adding 8.0 µl of 4 M NaCl and then 40 µl of autoclaved 13% PEG_{8000}. The mixture was incubated on ice for 20 min and then the plasmid DNA was pelleted by spinning for 15 min at 4°C, and washed with 70% ethanol. The DNA was then dried and dissolved in 20 µl of deionised H_2O and stored at -20°C.

2.2.10.2 Nested deletion of cloned DNA for sequencing

To sequence an over 1 kb DNA fragment, exonuclease was used to create smaller fragments. Unidirectional nested deletions of double-stranded DNA clones were obtained by using Exonuclease III and Mung Bean Nuclease in the "Exo-size™ Deletion kit" (New England Biolabs).

Exonuclease III is a 3'-exonuclease that is active only on double-stranded DNA at 5' overhang or blunt ends, while a 3'-overhang end, of three or more bases in length, is resistant to the enzyme. A 5' overhang site filled-in with α-phosphorothioate deoxynucleoside triphosphates is also resistant to the enzyme. It is therefore relatively easy to prepare linearized DNA with only one end that can be digested by exonuclease III to derive unidirectional nested deletions. After exonuclease III digestion, the resulting single-strand tails were digested by mung bean nuclease and then the DNA was religated and transformed. Because exonuclease III digestion occurs at a uniform rate it is possible to obtain progressively longer deletions, by removing aliquots of the deletion reaction at timed intervals. The practical steps are shown in Fig. 2.2.

To obtain deletions from a 1 Kb insert in 200-250 bp increments, 5-10 µg DNA was digested with appropriate restriction enzymes to create a unique 4-base 3' overhang (eg. in pBluescript with *Bst*XI, *Kpn*I, *Sac*I, *Pst*I) and a unique 5' overhang. The digestion was stopped by adding 1/10 volume of 0.2 M EDTA pH 8.0. The DNA was then purified by phenol/chloroform extraction and precipitated with ethanol as in 2.2.6. The DNA pellet was dissolved in 1 x exonuclease III reaction buffer and an aliquot was run on an agarose gel to estimate the concentration. About 1.25 µg DNA was incubated with 12.5 U exonuclease III at 37°C and 5 samples were taken at 40 sec intervals and digestion was stopped by heating at 70°C for 15 min. Each sample was then incubated with 3 U mung bean nuclease at 30°C for 30 min and the reaction was stopped by adding 1/10 volume of 0.2 M EDTA. Half of each sample was electrophoresed on an agarose gel to analyse the size of the deletion, and half of the sample was used for ligation, transformation, and colony analysis. The amount of exonuclease III used and the time of reaction were optimised for each source of DNA.

2.2.10.3 PCR-mediated DNA sequencing

For DNA sequencing four different fluorescent dye-labelled dideoxy nucleotides (G, A, T, C) were used in method of the dideoxy chain termination (Sanger et al., 1977). When these terminators replace standard dideoxy nucleotides in enzymatic sequencing, a dye label is incorporated into the DNA along with the terminating base. Products

differing in length by a single base therefore collectively demonstrate the sequence. PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems) was used.

The sequencing reaction was carried on a DNA thermal cycler "Perkin-Elmer Cetus Model 480" according to the manufacturer's protocol. The products were analyzed on the Applied Biosystems Model 373A Automated DNA sequencer, software version 1.2.0.

2.2.11 Screening a wheat cDNA library

A wheat cDNA library purchased from CLONTECH (Cat.#: FL1092a) was used to isolate full length cdc2-like genes of wheat. The mRNA source of this cDNA library was from 13-day seedlings of *Triticum aestivum* L. variety: Tam107 Hard Red Winter. The library was constructed from mRNA which had been completely denatured by methylmercuric hydroxide to release secondary structures, and cDNA synthesised from both oligo(dT)-primer and random-primer. An *Eco*RI linker, CCG GAA TTC CGG, was used for cloning the cDNA inserts into the *Eco*RI site of the vector λgt10 phage. The library was amplified once in host strain C600hfl.

2.2.11.1 Library plating and titering

The library was aliquated into 50 µl and stored at -70°C for long-term storage. To avoid repeated freeze/thaw cycles, one tube was used at a time and stored at 4°C for up to 6 months.

Bacteria strain C600hfl used for library plating was streaked onto a $MgSO_4$-free LB agar plate containing 15 µg/ml tetracycline and incubated at 37°C overnight. This master plate was stored at 4°C and re-streaked at 2-week intervals. From the master plate, a single colony was picked, streaked onto another $MgSO_4$-free LB plate with tetracycline and incubated at 37°C overnight to provide a working plate.

A single colony from the working plate was picked and inoculated in LB broth + 10 mM $MgSO_4$ + 0.2% maltose (without antibiotics), grown at 37°C overnight until the OD_{600} of the culture reached 2.0.

Serial dilutions of the library were prepared with 1 x lambda dilution buffer (SM buffer) which consisted of 0.1 M NaCl, 0.01 M $MgSO_4$, 0.035 M Tris-HCl pH7.5 and 0.01% gelatin. 100 µl of diluted λ suspensions were incubated with 200 µl of C600hfl overnight culture at 37°C for 15 min. The mixture was mixed with 3 ml of melted LB top agar (45°C) which consisted of LB broth, 10 mM $MgSO_4$ and 0.7% agarose, and immediately poured onto an LB + 10 mM $MgSO_4$ agar plate prewarmed to 37°C for at least 1 h prior to use. The plates were cooled to room temperature for 10 min and then incubated in an inverted position at 37°C for 7-8 h or until plaques appeared on the plate. The plaques were counted to determine the titer (pfu/ml).

2.2.11.2 Probe labelling

The random primer method was used to radioactively label a probe for screening the library. The probe was a 280 bp PCR product from wheat cDNA which has 62% homology to yeast *cdc2/CDC28* genes and 80%-90% homology to plant *cdc2*-like genes.

The DNA fragment was amplified by PCR using the cloned plasmid DNA as template. The product was purified after agar electrophoresis (2.2.9.1) and labelled by the random-primed method based on the hybridisation of a mixture of all possible hexanucleotides to the DNA to be labelled. The complementary strand is then synthesised from the 3' termini of the random oligonucleotide primer using Klenow enzyme. [α-^{32}P]dCTP present in the reaction is incorporated into the newly synthesised DNA strand.

50 ng denatured DNA was heated at 100°C for 10 min and incubated at 37°C for 30 min with 2 µl hexanucleotide mixture (Boehringer), 3 µl dNTP (dATP, dGTA, dTTP) each at 0.5 mM, 5 µl [α-^{32}P]dCTP (50 µCi, 3000Ci/mmol), 1 µl Klenow enzyme (2 U labelling grade) and H_2O added to a final volume of 20 µl. The reaction was stopped by adding 2 µl 0.2 M EDTA pH 8.0 and heating at 65°C for 10 min.

To assay the efficiency of the labelling, two methods were used as follow:

One method employed electrophoretic separation of incorporated from un-incorporated label, by electrophoresis of 1 µl of labelling reaction on an 8% denatured acrylamide gel, which consisted of 12 g Urea, 5 ml 40% acrylamide (38% acrylamide, 2% bisacrylamide), 1.25 ml 20 x TBE, 8.75 ml H_2O, 150 µl 10% ammonium persulphate and 10 µl TEMED. After the non-incorporated [^{32}P]dCTP was run off the gel, when the bromophenol blue dye loaded with the sample was within 5-10 mm of the bottom of the gel, an autoradiograph was taken of the gel for 30 min. The radioactively labelled DNA showed a size range between 60 bp and 280 bp.

Alternatively the labelling reaction was precipitated with ethanol, washed to remove non-incorporated [^{32}P]dCTP and the DNA was resuspended in 100 µl of TE buffer. 1 µ l of this DNA solution was taken to be counted in a liquid scintillation system (Beckman LS 3801). The specific activity of labelled probe was usually 1-1.5 x 10^9 cpm/µg.

Before use in hybridisation the labelled DNA was denatured by heating at 100°C for 10 min.

2.2.11.3 Plaque hybridisation

According to the titer determined in 2.2.11.1, plates with phage density of approximately 10,000 pfu per 90-mm plate were prepared as described in 2.2.11.1 for the library screening.

(1) Filter replicas:

The plates were chilled at 4°C overnight. A nylon membrane filter (Colony/Plaque Screen^{TM}, NEN Research Products, USA) was numbered first, placed onto the top agarose of the plate, marked in 3 asymmetric locations with an 18-gauge needle containing a little Indian ink. After 2-3 min the filter was carefully peeled off, floated with plaque side up on 0.8 ml of 0.5 N NaOH for 2 min to denature the DNA. The filter was blotted on Whatman 3MM paper, then floated on 0.8 ml of 1.0 M Tris-HCl pH 7.5 for 2 min, repeated once. The filter was dried in air and then baked at 80°C for 1-2 h to fix the DNA to the membrane. Before hybridisation, the filter was briefly rinsed in 2 x SSC buffer.

(2) Hybridisation:

The filters were incubated in prehybridisation buffer at 65°C for 3-4 h. Usually 3 ml of buffer was used per filter and 10 filters were hybridised at one time. The prehybridisation buffer consisted of 5 x SSC; 1% (w/v) blocking reagent (Boehringer) added from 10% sterile stock solution in Maleate buffer (100 mM Maleic acid, 150 mM NaCl, pH 7.5); 0.1% (w/v) N-lauroylsarkosine; 0.02% (w/v) SDS. After prehybridisation, the filter were transferred to hybridisation buffer which was 20 ml prehybridisation buffer added denatured probe, prewarmed at 65°C; and hybridised at 65 °C overnight (16-20 h).

The filter were first washed in 2 x SSC, 0.1% SDS at room temperature for 20 min; then in 1 x SSC, 0.1% SDS at 65°C 15 min twice, then in 0.2 x SSC, 0.1% SDS 65°C 15 min. Completeness of washing was tested by applying a monitor to a blank filter. If the count was higher than 5, another wash was performed in 0.1 x SSC, 0.1% SDS at 65°C for 15 min or more. This was a high stringency wash.

The filters were then dried in air and placed on a piece of Whatman 3 MM paper and autoradiographed at -70°C for 2 days using "Hyperfilm^{TM}-MP" (Amersham) X-ray film.

The film was developed and aligned with filters to find positive plaques. An agar plug containing the positive plaque and others was removed to 500 µl of SM buffer, replated to obtain 2000-2500 plaques on a 90 mm plate and rescreened. After 4-5 cycles of isolation and rescreening, a single, well-isolated plaque could be picked up for isolation the λ DNA.

A total of 2 x 10^6 plaques was screened.

2.2.11.4 Isolation of bacteriophage λ DNA from liquid lysate

Since commercially available agar often contains strong inhibitors of restriction enzymes, a liquid lysate method was used to isolate λ DNA.

A 2.5 ml C600hlf overnight culture was prepared and from this 500 µl was mixed with 1.5 x 10^6 pfu phage and incubated at 37°C for 20 min to allow infection. The mixture was then diluted into 50 ml prewarmed LB broth containing 10 mM $MgSO_4$ and 0.2% maltose in a baffle flask and incubated at 37°C with fast shaking. Every hour, 1 ml

of this culture was taken to measure the OD_{600}. In the first 4 h, the OD_{600} indicated linear increase in turbidity then in the fifth h lysis caused a sharp decrease in turbidity and the culture became clear and foaming.

Once the culture had lysed, 100 µl of chloroform was added and shaking was continued for 5 min. The culture was then supplemented with 2.9 g of NaCl (to 1 M) followed by 10 µl of DNase (10 µg/µl) and 10 µl RNase (10 µg/µl) to digest bacterial nuclei acids during incubation at room temperature for 1 h. The cell debris was spun down at 9000 rpm (using JA20 rotor of Backman J2-21M/E centrifuge) for 15 min. The supernatant was divided into two 25 ml aliquots which each received 2.5 g PEG_{8000} that was brought into solution by rotation of the sample. The λ DNA was then allowed to precipitate at 4°C overnight.

The DNA was spun down at 10,000 rpm (JA20 rotor, Backman J2-21M/E) for 20 min, and then dissolved in 1 ml SM buffer, divided into 500 µl portions in eppendorf tubes and treated with 1 µl of DNase (1µg/µl) and 1 µl of RNase (10 µg/µl) per tube for 1 h at room temperature. The DNA was extracted with chloroform, phenol, then phenol/chloroform, then chloroform. Finally the DNA was recovered by ethanol precipitation, washed with 70% ethanol and dissolved in 50-100 µl TE buffer for excision and sequence analysis.

2.2.11.5 Insert excision and sequencing

To recover insert DNA from isolated λ DNA an *Eco*RI digestion was performed since the library was inserted into the *Eco*RI site of λgt10. For one clone an internal *Eco*RI site was discovered in the insert DNA. To obtain an entire insert from this clone partial digestion of *Eco*RI was applied. 35 µl of λ DNA was diluted in 100 µl 1 x digestion buffer and dispersed into 5 tubes with tube 1 containing 30 µl, tubes 2 to 4 containing 20 µl and tube 5 10 µl. Tube 1 received 0.5 µl *Eco*RI (12 U/µl) was immediately mixed and 10 µl transferred to tube 2. The cycle of mixing and transfer to the next tube was repeated until all tubes contained 20 µl. These tubes with serial three fold dilutions of the enzyme were incubated at 37°C for 20 min, then the reaction was stopped by adding 1 µl of 0.5 M EDTA to each tube and heating at 85°C for 10 min.

The cooled mixture was run on 1% agarose gel and the band corresponding in size to uncut insert was purified as in 2.2.9.1.

The insert DNA was then subcloned into pBluescript SK+ vector, *Eco*RI site and sequenced as described in 2.2.10.

2.2.12 Northern hybridisation

10 µg of total RNA or 1-2 µg mRNA was separated on a formaldehyde/agarose gel in 1 x MOPS buffer which consisted of 0.02 M 3-[N-Morpholino]-propane-sulphonic acid, 5 mM sodium acetate pH 7.0 and 1 mM Na_2EDTA. The gel was 1% agarose in 1 x MOPS buffer with 6.3% (v/v) formaldehyde. The RNA sample was prepared in 25 µl solution containing 12.5 µl formamide, 4 µl formaldehyde and 1 x MOPS buffer, incubated at 65°C for 5 min, then mixed with 2.5 µl running buffer (50% glycerol, 1 mM EDTA pH 8.0, 0.25% bromophenol blue and 0.25% xylene cyanol FF) for loading.

After electrophoresis the gel was stained with ethidium bromide and photographed, then rinsed in water. The standard capillary blotting method (Southern 1975) was applied using nylon membrane Hybond-N+ (Amersham) and 20 x SSC as transfer buffer. The RNA was then fixed on the membrane by baking at 80°C for 2 h. The membrane was then prehybridised and hybridised in a buffer described in 2.2.11.3 modified by containing 50% formamide. Hybridisation was carried out at 50°C overnight.

The membrane was exposed in a PhosphorImager cassette for several days to 1 week. The image was scanned and quantitated by PhosphorImager (Molecular Dynamics) using Image Quant Software v 3.0.

If the filter was to be re-probed, probes were removed from the nylon membrane by immersing in a boiling solution containing 5 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.1% SDS, then holding it at 60°C for 20-30 min with shaking. The filter was then dried and autoradiographed to check that the probes had been removed. The filter could then be pre-hybridised and hybridised with a new probe.

2.2.13 Southern hybridisation

About 20 μg of genomic DNA at 0.04 μg/μl was digested with *Eco*RI, *Hind* III or *Bam*HI in the buffer appropriate to the enzyme at 37°C for 4-5 h. The digestion was stopped by heating and the DNA precipitated with ethanol. The DNA pellet was dissolved in 20 μl H_2O and 5 μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water), heated at 65°C for 10 min with the lid open to allow evaporation of any trace of ethanol. The DNA was electrophoresed in 0.8% agarose gel in 1 x TAE buffer at 30 mA overnight.

After electrophoresis the gel was stained with ethidium bromide, photographed and then soaked in 0.25 M HCl until the dye had changed colour, and left for an additional 10 min. The DNA was then denatured by shaking the gel in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 30 min, then neutralised in 1.5 M NaCl, 0.5 M Tris-HCl pH 7.2 and 1 mM EDTA for 2 x 15 min. DNA in the gel was then transferred for Southern blotting (Southern 1975) onto Amersham Hybond-N^+ nylon membrane. DNA was then fixed on the membrane by baking at 80°C for 2 h. The hybridisation procedure described as in 2.2.11.3.

2.2.14 Complementation of *S. cerevisiae cdc28* mutants

The capacity of two wheat *cdc2*-like genes to complement budding yeast *Saccharomyces cerevisiae cdc28* mutants was examined. Temperature sensitive mutants *cdc28-13* (*Matα ade1, his2, leu2-3,112, trp1-1a, ura3Δ*) was kindly supplied by Dr Helenna Richardson (The University of Adelaide, Australia); *cdc28-4* (*Matα ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3, can1-100, ssd1-d*) and *cdc28-1N* (*Matα ade2-1, his3-11,15, leu2-3,112, trp 1-1, ura3, can1-100, ssd1-d*)were kindly supplied by Dr. Heribert Hirt (University of Vienna, Austria). These mutants all grow at a permissive temperature of 28°C but were blocked at the restrictive temperature of 37°C. *S. cerevisiae* wild tyre W303-1B was kindly supplied by Dr Xinjie Chen (RSBS, the Australian National University).

2.2.14.1 Cloning of wheat *cdc2*-like genes into the yeast expression vector pYES2

E. coli / yeast shuttle vector pYES2 (Invitrogen) is a high copy episomal vector designed for inducible expression of recombinant proteins in *S. cerevisiae*. The yeast *GAL1* promoter and *CYC1* terminator flank the multiple cloning site of the vector, which allows inducible expression of recombinant proteins by provision of galactose. The *URA3* gene in the plasmid allows selection of transformants in host strains with a *ura3*$^-$ genotype by testing on prototrophic medium (Fig 2.3).

Wheat *cdc2*-like gene type A (*cdc2TaA*) was excised from plasmid pBluescript SK$^+$ by cutting with *Bsr*BI and *Xho*I. *Bsr*BI site is upstream of the transcription initiation site ATG of *cdc2TaA* and *Xho*I is a unique site at pBluescript SK$^+$ downstream of the *cdc2TaA* open reading frame. This fragment was subcloned into *Sma*I and *Xho*I in pBluescript SK$^+$ and then cloned into pYES2 at *Bam*I and *Xho*I. The resulting plasmid was called pYES2/cdc2TaA, and the *cdc2TaA* gene was at correct orientation relative to *GAL1* promoter (Fig. 4.1).

Since wheat *cdc2*-like gene type B (*cdc2TaB*) was an incomplete cDNA missing 13 amino acids at the carboxyl terminal, while the RACE product RACE-B had complete carboxyl terminal, a whole gene was assembled by ligating a portion of the RACE-B at the carboxy terminal of the *cdc2TaB* cDNA. The accuracy of combining two pieces of cDNA into a whole gene is discussed in Chapter 3 (3.2.3). Ligation was performed at the *Hind* III site, which is located at 423 bp from starting codon ATG. The resulting 1389 bp *cdc2TaB* cDNA had an *Eco*RI site at the both 3' and 5' end, and it was inserted into the pYES2 *Eco*RI site. The recombinant plasmid was called pYES2/cdc2TaB and the orientation was checked by restriction digestion. pYES2/cdc2TaB with sense-orientation relative to *GAL1* promoter was used for transformation of yeast cells (Fig. 4.2).

2.2.14.2 Yeast transformation by the lithium chloride method

A single colony of yeast mutants was grown in 3 ml YEPD medium (see Sppendix) at 28°C overnight. The culture was then diluted into 20 ml YEPD medium and growth continued for about 4 h until cells reached 5 x 10^7/ml, at which the OD_{600} was between 1-1.2. The cells were spun down at 3000 rpm for 5 min and resuspend in 500 µl of 50

mM LiCl, 1 x TE, 25 mM DTT (freshly mixed), incubated at 28°C for 30 min with shaking. About 5 µg of transforming plasmid DNA mixed with 40 µg of denatured calf thymus DNA was added to 100 µl of the cells and shaked vigorously for 30 min. The mixture was then supplemented with 700 µl of LiPEG (40% PEG_{3350}, 0.1 M LiCl and 1 x TE) and shaked for 30 min or more. Cells were heat shocked by incubating at 42°C for 5 min, then washed with water once. Cells were resuspended in 3 ml YEPD, incubated at 28°C for 1-3 h with shaking, and then plated on selective minimal medium without uracil (0.67% yeast nitrogen base, 2% glucose, 2% agar supplemented with 40 mg/l of each amino acid according to the auxotrophy of the yeast strain). The plate was incubated at 28°C for 2 days.

2.2.14.3 Complementation studies

The transformants were picked up from the Ura^- plate and streaked onto Ura^- galactose plate which consisted of 2% galactose instead of 2% glucose. The plate was first incubated at 28°C for 3-5 h for inducing transcription of the wheat *cdc2*-like gene, then transfered to restrictive temperature 37°C, incubated for 5 days. Any colonies rescued by wheat *cdc2*-like genes appeared at 3-4 days' incubation.

To test whether the complementation resulted from presence of the introduced plasmid, the cells harboring pYES2/cdc2TaA were grown in rich medium YEPD at 28°C overnight to relax selective pressure for retention of the plasmid. Then cells were plated on YEPD at 150-200 cells/plate. After incubation at 28°C for 2 days colonies appeared and replicas were made to test for uracil prototrophy. Replicas made by sterile velvet replica-plating were imprinted onto both Ura^- and Ura^+ plates. The colonies with plasmid (Ura^+) judged by ability to grow without uracil supplementation, and colonies without plasmid (Ura^-) were restreaked onto a Ura^+ galactose plate, incubated at 37°C for 2 days to test capasity for division at restriction temperature.

2.2.14.4 Test of cell proliferation in liquid culture

To investigate the capacity for cell proliferation in yeast mutants rescued by wheat *cdc2*-like gene, cells were grown at 28°C in glucose selective medium (Ura^-) for one and

half days. The cells were then washed twice with water and once with Ura$^-$ galactose medium, then resuspended in galactose medium and grown at 28°C for 1 h. The cell density was then counted using a hematocytometer and cells were diluted to 4 x 10^6/ml in 3 ml galactose medium which was prewarmed at 37°C. Samples during growth at 37° C were taken at 0, 6, 12, 24, 36, 48 h, sonicated (5 sec) immediately to disassociate sticky cells that had completed the cell cycle, placed on ice and cell number was counted freshly using a hematocytometer.

2.2.14.5 Flow cytometric analysis of cell proliferation

Cells were grown in liquid culture and sampled as described in 2.2.14.4. Culture samples were immediately spun at 1500 *g* for 5 min, then washed in 50 mM Tris-HCl pH 7.5 buffer once and fixed in 70% ethanol for 1 h at room temperature. The fixed samples could be stored at -20°C for several days. For flow cytometric analysis fixed cells were spun down and washed in 50 mM Tris buffer as above, then treated with 0.1% RNase solution (100 mg in 100 ml 50 mM Tris buffer) for 1 h at 37°C. The cells were then spun down and resuspended in pepsin solution for 15 min at room temperature. Pepsin solution was prepared by dissolving 0.5 g pepsin (1000 Units/g MERCK) in 5.5 ml 1 N HCl and then diluting to 100 ml with distilled water. After pepsin treatment cells were spun down and resuspended in PI (propidium iodide) solution and kept in dark for 1 h at room temperature. PI solution was prepared by dissolving 5 mg propidium iodide (CALBIOCHEM) and 1.42 g $MgCl_2 \cdot 6H_2O$ in 100 ml Tris buffer that was 0.18 M Tris-HCl and 0.19 M NaCl, pH 7.5. After PI staining cells were spun down and washed in 50 mM Tris buffer, finally resuspended in 50 mM Tris buffer and run in a FACScan (BECTON DICKINSON).

2.2.14.6 Western blot analysis of wheat *cdc2*-like protein expressed in yeast cells

Yeast mutant *cdc28-13^{ts}* harbouring pYES2/cdc2TaA or pYES2/cdc2TaB was grown in medium with 2% galactose and no added uracil for two days at 28°C to allow expression of wheat Cdc2TaA or Cdc2TaB protein. Cells were then spun at 1500 g for 5 min and washed in water once and the pellet was frozen immediately in liquid nitrogen

and ground with glass beads to a fine powder. Proteins were extracted by mixing of 0.2 g grindate with 100 µl RIPA buffer (see 2.2.3.1) and 100 µl of SDS sample buffer (see 2.2.3.3), vortexing twice for 20 sec, heating in boiling water for 5 min. The mixture was spun at full speed in a microfuge for 5 min and supernatant was removed to a clean tube and ready for SDS gel electrophoresis as described in 2.2.3.3.

Proteins were Western blotted and immuno-detected by anti-PSTAIR antibody or anti Cdc2TaB antibody as described in 2.2.3.4.

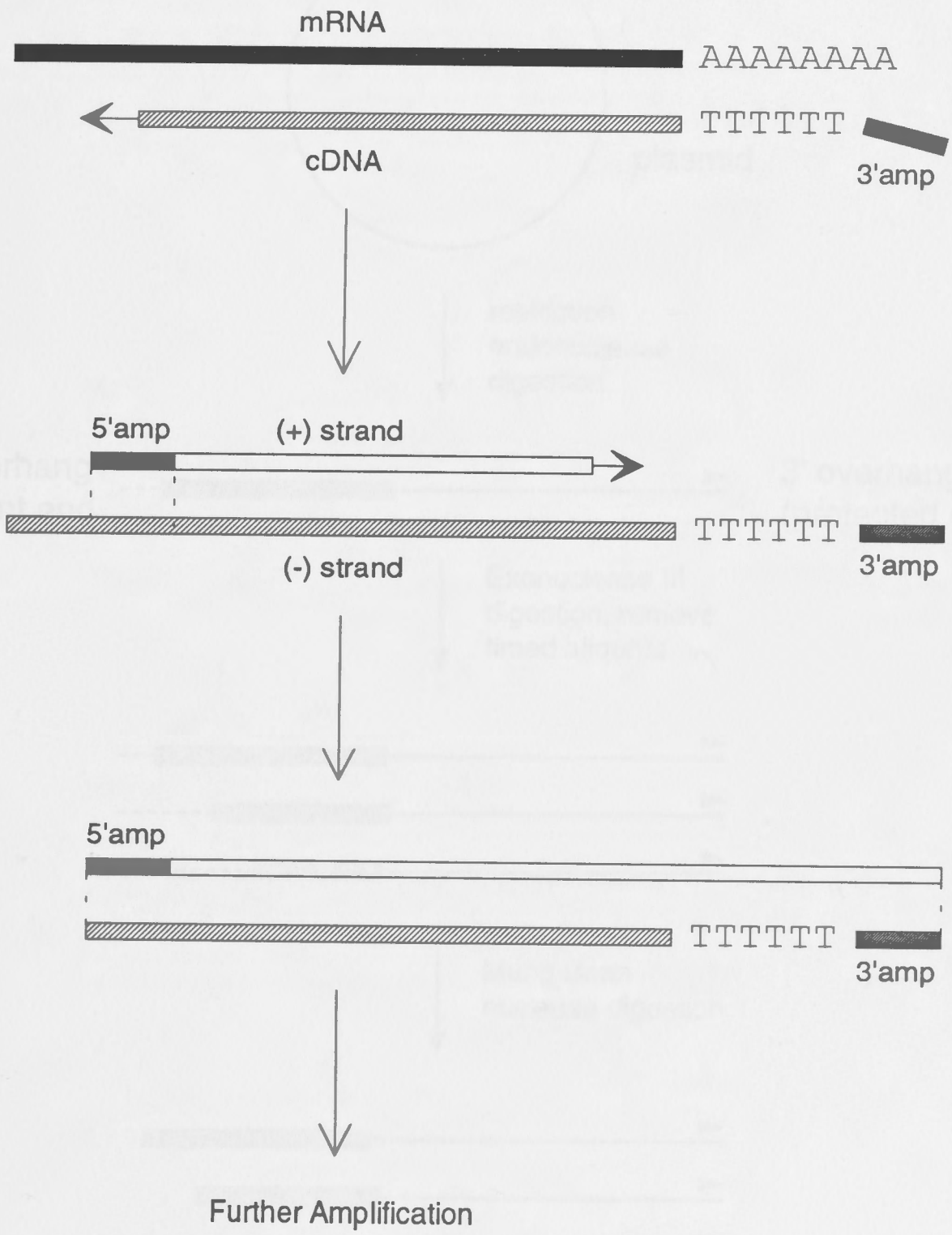


Fig. 2.1 Rapid Amplification of cDNA Ends (RACE, adapted from Frohman et al., 1988). The method described here makes it possible to amplify 3' ends of cDNAs. The reverse transcription (upper diagram) is carried out with the use of oligo(dT) primer or oligo(dT) flanked by a restriction site linker as 3' primer (3'amp). Then PCR amplification is performed with the use of 5'amp primer (which derives from a portion of the gene of interest isolated as initial PCR product) and 3'amp primer.

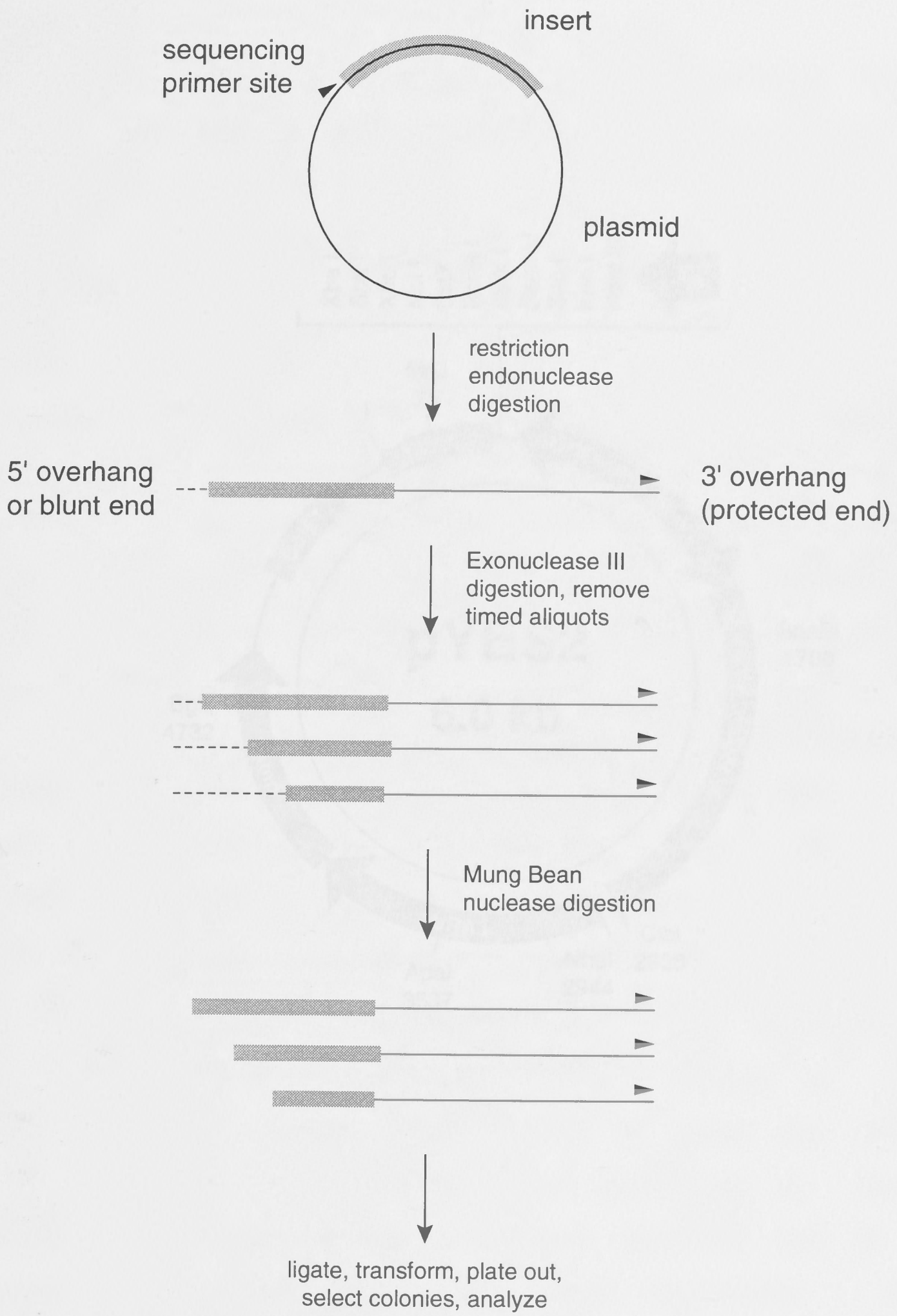


Fig. 2.2 Flow chart of nest deletion of recombinant plasmid DNA using exonuclease III and mung bean nuclease digestion.

▒▒▒▒: insert; ————: plasmid; ---------: single strand DNA

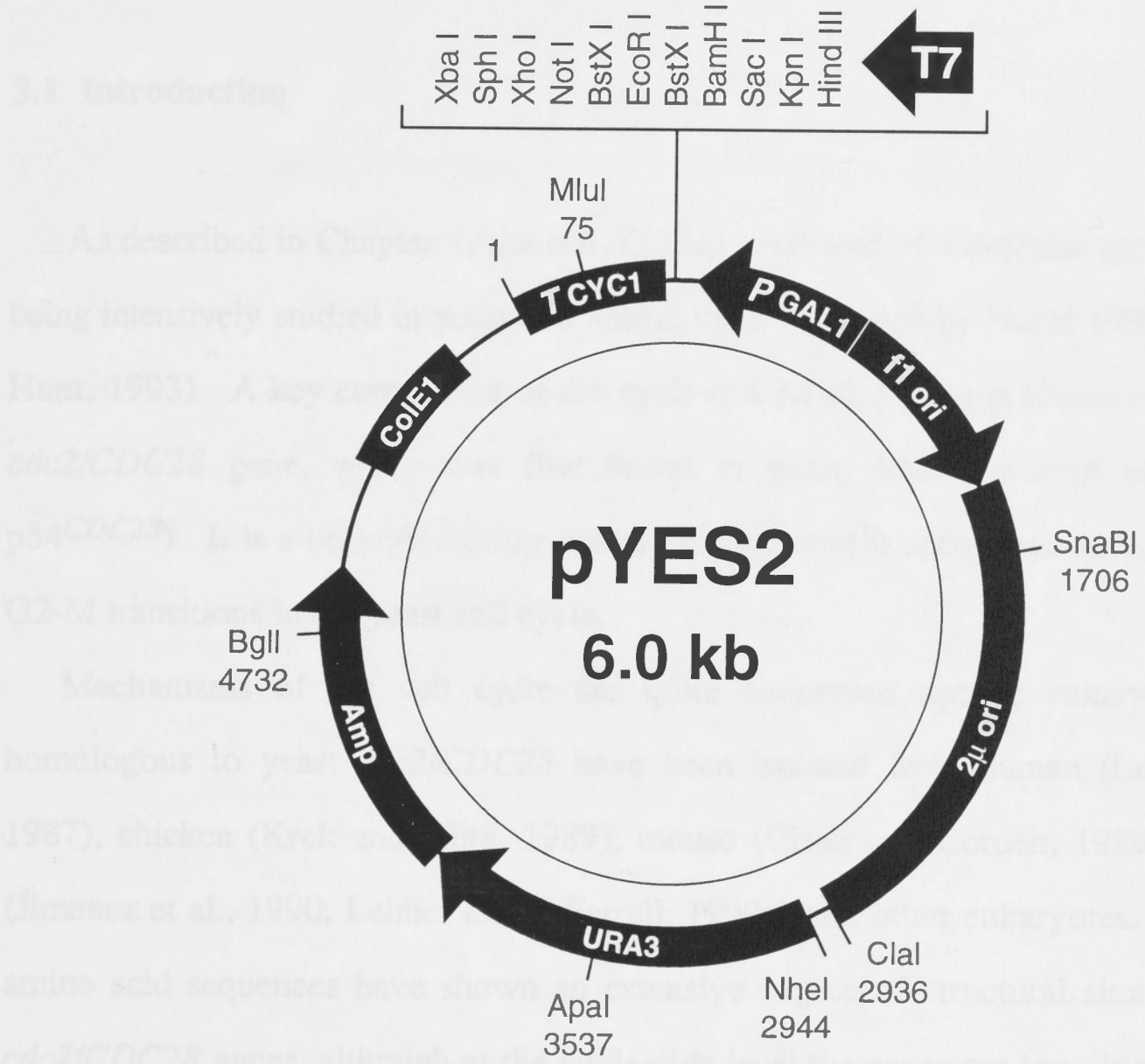


Fig. 2.3 Yeast expression vector.

Chapter 3

Molecular Cloning of Wheat *cdc2*-like Genes and Their Expression Correlating with Cell Proliferation

3.1 Introduction

As described in Chapter 1, the cell division cycle and its molecular mechanisms have being intensively studied in yeast and animal cells (reviewed by Nurse 1990; Murray and Hunt, 1993). A key component of the cycle is a 34-kDa protein kinase encoded by the *cdc2/CDC28* gene, which was first found in yeast, and is known as $p34^{cdc2}$ (or $p34^{CDC28}$). It is a serine/threonine protein kinase, whose activity governs the G1-S and G2-M transitions in the yeast cell cycle.

Mechanisms of the cell cycle are quite conserved among eukaryotes. Genes homologous to yeast *cdc2/CDC28* have been isolated from human (Lee and Nurse, 1987), chicken (Krek and Nigg, 1989), mouse (Cisek and Corden, 1989), *Drosophila* (Jimenez et al., 1990; Lehner and O'Farrell, 1990a) and other eukaryotes. The encoded amino acid sequences have shown an extensive degree of structural similarity to yeast *cdc2/CDC28* genes, although at the nucleotide level the genes are too divergent to allow detection by cross hybridisation (Beach et al., 1982). This is consistent with an evolution of the gene early in the development of eukaryotes and with the conservation of key functional regions that are indispensable for cell cycle progress, while at the nucleotide level codon usage has varied by genetic drift. Some of the similarities between *cdc2* genes are shared with members of the serine/threonine protein kinase family (Hanks et al., 1988) and are presumably concerned with the protein kinase function, while others are unique to *cdc2*-like kinases, including a perfectly conserved 16-amino acid region, the so-called "PSTAIR" domain.

In yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, *cdc2* and *CDC28* functions are required at both major control points, the G1-S and G2-M

transitions (Nurse and Bisset, 1981; Reed, 1980; Piggott et al., 1982; Reed and Wittenberg, 1990). However, higher eukaryotes have been found to contain multiple *cdc2*-like genes, whose products are collectively designated CDK (for cyclin dependent kinase) family. CDKs have different roles and in higher eukaryote cells many are active in G1 or S phase with Cdc2 retaining its importance at mitosis. In this terminology Cdc2 would be designated Cdk1 but most authors retain the name Cdc2 and this thesis will follow that usage. The best characterised CDK is Cdk2, a closely related kinase to Cdc2, whose function is required for G1-S transition and through S phase in human and *Xenopus* cells (Elledge and Spottswood, 1991; Paris et al., 1991; Fang and Newport, 1991; Tsai et al., 1993). Other Cdc2-related protein kinases in human cells are believed to function in G1 phase. Some are directly involved in signal transduction following growth hormone stimulation and are therefore better understood as involved in stimulating growth and promoting the G0 to G1 phase transition (reviewed by Sherr 1994; Pines 1995).

In higher plants, an indication of $p34^{cdc2}$ presence was obtained by antibody detection and evidence of cell cycle dependent phosphorylation in the unicellular plant *Chlamydomonas* by John et al. (1989), and its presence was rigorously confirmed by isolation of several *cdc2*-like genes in alfalfa (Hirt et al., 1991, 1993), maize (Colasanti et al., 1991), *Arabidopsis* (Ferreira et al., 1991; Hirayama et al., 1991), rice (Hashimoto et al., 1992) and soybean (Miao et al., 1993); *cdc2*-like gene fragments have been reported in pea (Feiler and Jacobs, 1990) and petunia (Bergounioux et al., 1992). These *cdc2*-like genes all show structural homology with yeast and animal *cdc2* genes and some of them have been shown to be functional homologues by complementation of *cdc2/CDC28* mutations in fission yeast or budding yeast. These results provide evidence that the basic mechanisms that regulate cell division in plants are likely to be similar to those in animals and yeasts, in which $p34^{cdc2}$ plays a central role. However the presence of Cdk2 in plants is not established.

Besides a number of *cdc2*-like genes, several cyclin-like genes have also been detected in plants (Hata et al., 1991; Hirt et al., 1992) and in some cases their expression in cell division regions is consistent with involvement in cell proliferation (Hemerly et al.,

1992; Ferreira et al., 1994a; Fobert et al., 1994; Sauter et al., 1995). The available evidence therefore suggests that the basic mechanisms of the cell cycle in plant cells is similar to that in other eukaryotes (reviewed by Staiger and Doonan, 1993; John et al., 1993a).

Despite these similarities, the cell cycle regulators might respond to different developmental programs in the plant and animal kingdoms. Most plant morphogenesis is achieved by continual iterative development at the apical meristem rather than largely restricted to embryogenesis as in animals. Plant hormones are small non-protein molecules unlike the peptide growth factors of higher animals, and also the extensive totipotency of plant cells, may indicate that the interaction of hormones with the cell cycle differ between plants and animals and therefore the roles of CDKs involved in G1 phase may also differ. The observation that somatic plant cells can depend on a specific hormone (cytokinin) for initiation of mitosis (Zhang et al., 1996) indicates that hormones can interact with the plant cell cycle at points different from those in somatic animal cells (see final Discussion). Thus, the developmental controls in plants must be integrated with mechanisms confining cell division activity to the meristem and imposing cessation of division as cells leave the meristem and differentiate in formation of highly ordered plant organs. Our laboratory obtained the first evidence that the cessation of division may be enforced by low levels of p34^{cdc2} in cells leaving the meristem (John et al., 1990) and the significance of this for cell division is underlined by the necessity for restoration of the level of p34^{cdc2} at resumption of division (Gorst et al., 1991; John et al., 1993b). To further study the involvement of *cdc2*-like genes in plant cell proliferation, and in the switch to cell differentiation during the course of development, I attempted to clone wheat *cdc2*-like genes, since the first evidence for preferential expression of *cdc2* in a plant meristem was obtained in wheat (John et al., 1990).

Four strategies have been used in other laboratories to clone homologues of *cdc2* genes: (1) complementation of yeast *cdc2/CDC28* mutants to detect genes with similar functions, by this approach human *cdc2* and *cdk2* were isolated (Lee and Nurse, 1987; Elledge and Spottswood, 1991, Ninomiys-Tsuji et al., 1991); (2) low stringency hybridisation to detect nucleotide sequences shared with genes from taxonomically close

relatives, by this method chicken *cdc2* and two *Arabidopsis cdc2* genes were isolated (Krek and Nigg, 1989: Hirayama et al., 1991); (3) antibody screening of expression libraries to detect shared structural features, a probable pea *cdc2* homologue was obtained by immunological screening of PCR products in a bacterial expression vector (Feiler and Jacobs, 1991); (4) PCR amplification of a highly conserved region followed by screening a library using the amplified sequence as a probe; by this strategy *Drosophila cdc2* (Lehner and O'Farrell, 1990) and most plant *cdc2* genes were isolated (Ferreira et al., 1991, Hirt et al, 1991, 1993; Colasanti et al., 1991; Hashimoto et al., 1992; Miao et al., 1993).

I have used the fourth of these strategies for cloning wheat *cdc2*-like genes. The extensive structural similarity of p34^{cdc2} homologues among yeast, animal and plant cells (see Fig. 3.12) allowed me to design degenerate oligonucleotides for priming PCR amplification of DNA that contained sequences conserved in *cdc2* genes. It is possible to isolate genes or gene families related to *cdc2* by this method. In this chapter I describe the isolation and characterisation of cDNA clones encoding p34^{cdc2}-like sequences from wheat, and the expression of these genes in tissue containing proliferating or non-proliferating cells.

3.2 Results

3.2.1 Isolation of two wheat *cdc2*-like gene fragments by RT-PCR

Lee and Nurse (1987) first noted the remarkable amino acid sequence conservation in the p34^{cdc2} kinase of highly diverse species. Fig. 3.12 is an alignment of amino acid sequences of variant *cdc2* genes from *S. pombe*, *S. cerevisiae*, human, *Arabidopsis*, alfalfa, rice and maize, showing regions of identity at the amino acid level that are common to all *cdc2* genes isolated so far. Some regions are concerned with ATP binding and phosphate transfer, for example the amino acid sequence GEGTYGVV near the amino terminus of the protein is an ATP-binding domain, which in other protein kinases is identified as GXGXXGXV (Hanks, et al., 1988). Another highly conserved

region, at about the 130th amino acid, HRDLKPQN is a protein kinase domain that contains several residues also conserved among many protein kinases. Other regions of the protein are concerned with the specific functions of CDKs. A specific motif for Cdc2 and Cdk2 is the region containing 16 amino acids, EGVPSTAIREISLLKE at around the 50th amino acid, (the PSTAIR region) which is believed to be a mitotic cyclin binding site. Degenerate oligonucleotide primers complementary to these highly conserved regions were designed, as described in Table 3.1. 5'cdc2/I primer and 3'cdc2/I was a pool of all possible oligonucleotide sequences encoding the amino acid sequences EGTYGVVY and HRDLKPQN respectively. Another 5' primer, 5'cdc2/II, encoded EGVPSTAI but was less degenerate than 5'cdc2/I and 3'cdc2/I because based on nucleotide sequences of rice and maize *cdc2* genes according to Hashimoto et al. (1992) and Colasanti et al. (1991), thus potentially specific for monocotyledonous *cdc2* homologues. Another 3' primer 3'cdc2/II encoded the region around the 170th amino acid of the protein, which has the sequence WYRAPEI/VLL that is a highly conserved general protein kinase catalytic domain. Although in animal *cdc2* V replaces I in this stretch, ie, WYRAPEVLL, all known plant *cdc2* genes contain I rather than V, therefore primer 3'cdc2/II was designed to encode WYRAPEILL and combined the sequences of rice and maize in this region.

The total RNA used in reverse transcriptase-PCR (RT-PCR) was isolated from wheat leaf meristem, in which the *cdc2* gene product accumulates preferentially (John et al., 1990). The method of RT-PCR was described in Chapter 2 (2.2.8.2). The results of RT-PCR are showed in Fig. 3.1. When 5'cdc2/I and 3'cdc2/II were used as primers, a very faint band of about 500 base pair could be observed (lane 1 and lane 2 in Fig. 3.1). Products of this reaction could encode a number of protein kinases. To improve the sensitivity and specificity, the RT-PCR was followed by nested PCR in which one of the primers was specific for *cdc2* and not other protein kinases. Lanes 3 and 4 of Fig. 3.1 show the results of the nested PCR in which 5'cdc2/II and 3'cdc2/I were used as internal primers and 1/25 volume of primary RT-PCR was used as template. The resulting 280 bp band was precisely the length predicted to occur in the *cdc2* gene between the regions at which primers 5'cdc2/II and 3'cdc2/I are expected to bind.

The 280 bp PCR product was isolated and cloned into pBluescript SK+ vector at *EcoRI* and *BamHI* sites then transformed to *E. coli* DH5α strain. 30 positive colonies were picked and the inserts in plasmids from 8 colonies were sequenced. Of these, 7 were identical and encoded amino acid sequences similar to other *cdc2* genes; this sequence was named type A. One of the colonies contained a sequence different from the other seven, but still similar to other *cdc2* genes, and was named type B. The sequences and their deduced translation products are shown in Fig. 3.2.

Both amino acid sequences, deduced from the two types of putative *cdc2* gene, revealed similarity with other *cdc2* genes. Type A was 94% identical to the *cdc2* homologue of the monocotyledonous plants maize and rice; and also 65% and 62% identical respectively to human *cdc2* and yeast *S. cerevisiae CDC28*. Type B was 82% identical to maize and rice *cdc2* and 65% identical to human *cdc2* and yeast *S. cerevisiae CDC28*. Type A and Type B shared 82% identity with each other. The regions of greatest difference between the two types of wheat gene were confined to regions that are not conserved among known $p34^{cdc2}$ homologues. This suggested that type A and type B were derived from two separate genes, both potential homologues of the *cdc2* gene. To test this homology the complete genes were sought.

3.2.2 Amplification of the 3'end of wheat *cdc2*-like cDNAs

The availability of PCR-amplified portions of wheat *cdc2*-like genes made it possible to amplify the rest of the gene by PCR using the method of RACE (rapid amplification of cDNA ends) described by Frohman et al. (1988) (see Methods, 2.2.8.3). Two unique oligonucleotides, 5'cdc2A and 5'cdc2B (shown underlined in Fig. 3.2), based on the sequences of type A and type B *cdc2*-like PCR products, were synthesised as forward primers and each was flanked with a *BamHI* site at the 5' end. The 3' primer was oligo$(dT)_{17}$ with a flanking sequence of *EcoRI* site and was called 3'dT_{17}RI.

First-strand cDNA was synthesised as in RT-PCR (see Methods 2.2.8.2). The direct amplification of 3' cDNA ends using 3'dT_{17}RI and 5'cdc2A or 5'cdc2B as primers was not successful, therefore the method of nested PCR was employed. A first reaction was

performed using 5'cdc2/II and 3'dT$_{17}$RI as primers and then a nested PCR was performed by using 5'cdc2A or 5'cdc2B and 3'dT$_{17}$RI as primers. Two bands, about 800 bp and 1 kb, were obtained in nested PCR using 5'cdc2A and 3'dT$_{17}$RI as primers, and were called RACE-A1 and RACE-A2 (Fig. 3.3A). When 5'cdc2B and 3'dT$_{17}$RI were used as primers in the nested PCR one dominant band of 1 kb was obtained, and was called RACE-B (Fig. 3.3B).

These three RACE products were cloned into pBluescript SK$^+$ plasmid cut with *EcoRI* and *BamHI* and the inserts were fully sequenced. This analysis revealed that the 800 bp fragment RACE-A1 comprised an extension of the type A fragment to the poly(A) tail of the cDNA. The 1 kb fragment RACE-A2 was a false product not related to *cdc2*, however the 1 kb fragment of RACE-B was an extension of fragment type B to the poly(A) tail of the cDNA. The sequences and deduced amino acid sequences of RACE-A1 and RACE-B are shown in Fig. 3.4 and Fig. 3.5. Another clone of 800 bp fragment, called RACE-A1*, was sequenced and showed 6 nucleotides different from that of RACE-A1 in the coding region, and in the 3' noncoding region they were also very similar to each other (Fig. 3.6). These two type A fragments shared 98.4% identity in nucleotide sequence and they might represent alleles or homoeologues of the type A gene (see discussion in this Chapter).

The extended sequences of RACE-A1 and RACE-B showed strong similarity with other known *cdc2* genes (see Fig. 3.4 and Fig. 3.5). A comparison between the overlapping region of 280 bp type A fragment and RACE-A1 showed one nucleotide difference at the 22rd position of RACE-A1, where a G substituted an A in the 280 bp type A fragment. Also in RACE-B, there was one nucleotide difference at the 50th position, where a T substituted a G in the 280 bp type B fragment therefore changing the codon to L from R. These differences may represent the inherent error in PCR amplification. To obtain a full length copy free from errors induced by PCR a cDNA equivalent to each gene was sought.

3.2.3 Screening a wheat cDNA library revealed two *cdc2*-like genes

To isolate full-length cDNAs, the 280 bp PCR-fragment of type A was used to probe a cDNA library of 13-day-old seedling wheat (*Triticum aestivum* L. variety: Tam107 Hard Red Winter, purchased from CLONTECH). Because the 280 bp type A and Type B DNA fragments can cross-hybridise with each other (Fig. 3.7), it is possible to detect cDNA corresponding to both genes when using only type A as probe to screen the library. Five positive signals were identified from 2 x 10^6 phages screened, and these were named clone 4, clone 5, clone 8, clone 9 and clone 10 (shown in Fig. 3.8). Phage λ DNA was isolated from these positives and insert cDNA was excised by *EcoRI* digestion and subcloned into pBluescript SK^+ (Methods 2.2.11.4 and 2.2.11.5). Digestion revealed that clone 4 contained a 900 bp insert, clone 5 and clone 8 contained a 1.5 kb insert, clone 9 contained 1.2 kb insert with an internal *EcoRI* site that divided the insert into 500 bp and 700 bp fragments, and clone 10 contained a 800 bp insert. Sequences of both ends of these five clones revealed that clone 4 and clone 10 were identical to type B, and clone 5, clone 8 and clone 9 were identical to type A. Clone 9 contained a near full-length cDNA of type A, but clone 5 and clone 8 contained only the half of the gene near its N-terminus. Therefore I chose clone 4 and clone 9 for sequencing in both directions.

Sequence analysis of these clones revealed extended open reading frames and the putative proteins encoded by these open reading frames all have sequence similarity to $p34^{cdc2}$ kinase (see red boxes in Fig. 3.12). These two *cdc2*-like cDNAs were therefore tentatively named *cdc2TaA* (clone 9) and *cdc2TaB* (clone 4); *Ta* represents *Triticum aestivum*. The DNA sequences and deduced amino acid sequences are shown in Fig. 3.9 and Fig. 3.10 (GenBank accession no. U23409 and U23410). The cDNA of *cdc2TaA* contained an 882-bp open reading frame from position 216 to 1097, encoding a protein of 294 amino acids. *cdc2TaB* contained a near-complete open reading frame but lacked 13 amino acids at the carboxyl terminal that are known from the complete sequence in this region determined in RACE-B (Fig. 3.5).

Sequences of *cdc2TaA* and *cdc2TaB* showed they were 79.7% identical at the nucleotide level and 85% identical at the amino acid level. The greatest difference between *cdc2TaA* and *cdc2TaB* was in regions of the gene that have been less conserved by evolution and may therefore not be functionally significant (Fig. 3.11A and Fig. 3.12). A search of the GenBank and EMBL databases for both genes showed that each was similar to *cdc2*-related genes, especially to maize and rice *cdc2* genes. All highly conserved regions in known *cdc2* proteins are also present in both wheat *cdc2*-like genes. For example, there is absolute conservation in the wheat genes of the ATP-binding-site motif GEGTYGVV (amino acids 11-18), the 16 amino acid PSTAIR domain (amino acids 42-58), and domains typical of protein kinases, eg, HRDLKPQN, 125-132 in *cdc2TaA* and 124-131 in *cdc2TaB*; DFG, 146-148 in *cdc2TaA* and 145-147 in *cdc2TaB*. The amino acids Thr14, Tyr15 and Thr161, which are subject to phosphorylation/dephosphorylation in regulation of p34^{cdc2} activity, were also present in both wheat *cdc2*-like genes. Fig. 3.12 shows an alignment of wheat *cdc2* genes with other plant *cdc2* homologues and yeast *cdc2*, *CDC28*, human *cdc2*, as well as *cdk2* genes from human, *Xenopus* and goldfish. Both wheat *cdc2*-like genes retain all these conserved region (red boxes). It is therefore most likely they are closely related but distinct (non allelic) *cdc2*-like genes of wheat.

A comparison of the wheat *cdc2* sequences with published amino acid sequences of *cdc2*-homologous is shown in Table 3.2. This figure assembles a complete amino acid sequence for Cdc2TaB by adding 13 amino acids to the carboxyl-terminal as were determined from the extensively-overlapping and unambiguously-identical RACE-B; the validity of this derivation of the terminal amino acids is discussed below. *cdc2TaA* is similar to monocotyledonous *cdc2* homologues with 92.5% identity to rice and maize *cdc2a* genes. There is also 82-85% identity with dicotyledonous *cdc2* genes and 60-63% identity to animal and yeast *cdc2* genes, 66% to *cdk2* genes. Similarly *cdc2TaB* resembles plant *cdc2* genes, with about 84-92% identity, and also 60-63% identity to animal and yeast *cdc2* genes, and 63% to *cdk2* genes. These strong similarities further indicate that *cdc2TaA* and *cdc2TaB* are possible *cdc2* homologues. Comparing wheat *cdc2* genes with rice *cdc2* genes, interestingly, *cdc2TaA* is very similar to rice *cdc2-1*

with 92.5% identity and only 84.1% similarity to rice *cdc2-2*, while *cdc2TaB* is very similar to rice *cdc2-2* with 91.8% identity and only 83.7% similarity to rice *cdc2-1*. Thus a pair of *cdc2*-like genes may be a common feature in cereals.

To assess the accuracy with which the last 13 amino acids of RACE-B indicate the missing sequence in *cdc2TaB*, I compared the sequence of *cdc2TaB* with that of RACE-B, which also provided information concerning whether they are derived from the same gene, or perhaps different alleles of the same gene, or from two different *cdc2* genes. Also I did the same comparison between *cdc2TaA* and RACE-A1. In *cdc2TaA* and RACE-A1 (526-1170 in Fig. 3.9 and 22-667 in Fig. 3.4), 646 nucleotides overlapped (excluding the primer regions), and the nucleotide sequences were identical except the first nucleotide in RACE-A1 was G instead of A in *cdc2TaA*, which changes the codon to read V instead of I. This one nucleotide difference is probably induced by the inaccurate PCR amplification of RACE-A1, because this nucleotide in the initially obtained 280-bp type A sequence (see Fig. 3.2A) is also an A rather than a G. It is therefore concluded that *cdc2TaA* and RACE-A1 are derived from the same gene. When I compared the *cdc2TaB* (Fig. 3.10) with RACE-B (Fig. 3.5), I observed in the 534 nucleotide overlapping region (382-915 in Fig. 3.10 and 22-555 in Fig. 3.5, excluding the primer regions) 11 nucleotide differences. Among these only one is predicted to cause an amino acid exchange; where the nucleotide A in 410th position of *cdc2TaB* (Fig. 3.10) is replaced by T in RACE-B, so changing the codon to read H instead of R (the 112th amino acid, see Fig. 3.12), both H and R are commonly found in this position in other plant *cdc2* genes (Fig. 3.12). The other 10 nucleotide exchanges occurred in the third codon position so they do not change the encoded protein sequence. It is therefore most likely that *cdc2TaB* and RACE-B are derived from the same gene but may not be identical because they may be homoeologues derived from different parent genomes that contributed to hexaploid wheat. In fact the overlapping amino acid sequences of *cdc2TaB* and RACE-B (178 amino acids) share 99.5% similarity, so it is valid to add the last 13 amino acids of RACE-B to the missing C-terminal of *cdc2TaB*.

Unexpectedly, both wheat *cdc2*-like cDNAs contain GC-rich leader sequences upstream of the start codon ATG. *cdc2TaA* and *cdc2TaB* have 76.3% and 88% GC content at the 5' noncoding regions respectively. This could indicate that the translation of both genes is slow or can be regulated (Kozak, 1991b). Moreover, the 5' region of *cdc2TaA* contains a 24 codon upstream open reading frame (Fig 3.9 from nucleotide 33 to 104). The two open reading frames (of 24 codons and 294 codons) are separated by 108 nucleotides. This is a rare feature in eukaryotic mRNAs. It is probable that the 5' noncoding sequences of *cdc2TaA* represents an mRNA precursor, alternatively, it may be a functional mRNA in which the upstream open reading frame could play a role in controlling translation of the *cdc2TaA* gene (see Discussion in 3.3.3).

3.2.4 Expression of wheat *cdc2*-like genes parallels cell proliferation

Northern blotting was used to determine the transcript size of the wheat *cdc2*-like genes and possible differential accumulation of transcripts in different tissue regions. Total RNA, then mRNA, was isolated from wheat root meristem and separated on 1.2% agarose gel containing formaldehyde (see Methods in 2.2.7 and 2.2.12). Hybridisation with the 280-bp PCR-fragment type A revealed an mRNA size of about 1.5 kb (see Fig. 3.13A). Because the 280-bp PCR-fragment typeA can hybridise with both typeA and typeB clones (Fig. 3.7), it could detect both transcripts. A single mRNA band of around 1.5 kb was detected (Fig. 3.13A), which is the size of *cdc2* transcript also found in rice, alfalfa and *Arabidopsis* (Hashimoto et al., 1992; Hirt et al., 1993; Hirayama et al., 1991). It is possible that two transcripts are close to each other at about 1.5 kb. Two bigger bands at about 3.5 kb and 6.5 kb presented in lane 3 of Fig. 3.13A were probably due to impurities causing aggregation of mRNA or contamination, because the mRNA used in lane 3 was directly isolated from wheat tissue without first obtaining protein-free total RNA.

In yeasts, *cdc2* mRNA level does not vary during the cell cycle (Durkacz et al., 1986) and therefore p34^{cdc2} does not appear to be regulated at the transcriptional level. In multicellular organisms that contain cells in various developmental states, from rapidly dividing to quiescent, the expression of the *cdc2* gene is transcriptionally regulated (Krek and Nigg, 1989; Lee et al., 1988; Colasanti et al., 1991; Hirt et al., 1993; John et al., 1993b). Previous results have shown that high p34^{cdc2}-like protein levels (PSTAIR-containing protein) do correlate with the location of actively dividing cells in wheat leaf (John et al., 1990). To investigate whether the *cdc2* transcript level correlated with cell division activity in plant tissue, total RNA was isolated from (1) wheat root tip and leaf meristem where cells are dividing actively; (2) from the middle segment (30-40 mm from the base) of 7-day-old leaf, where cell division is stopped and cells are undergoing differentiation; (3) 7-day-old leaf tip region (80-90 mm from the base) where cells are differentiated, and (4) wheat suspension culture cells which were proliferating. 20 µg of each total RNA was separated on 1.2% agarose gel containing formaldehyde, Northern blotted, and then hybridised with 280-bp type A fragment. A 1.5 kb messenger RNA was abundant in those tissues that contained actively dividing, undifferentiated cells, such as root tip, leaf meristem and suspension cultured cells (Fig. 3.13B). However wheat *cdc2* mRNA was almost completely absent from differentiating tissue, such as leaf middle segment, and from terminally differentiated tissue, such as leaf tip. As a control, the same filter was reprobed with a 18S rRNA gene fragment (wheat rDNA isolated by Appels and Dvorak, 1982) as shown in Fig. 3.13B (b). All RNA showed similar 18s rRNA levels. This indicates that the total RNA from various tissues was equally loaded and failure of detection of *cdc2* transcript in segments from middle and tip of leaf was due to low levels of *cdc2* mRNA relative to other species of RNA. This result is consistent with the previous finding of high p34^{cdc2}-like protein level only in actively dividing cells of wheat leaf (John et al., 1990) and indicates that the restricted accumulation of the protein may be regulated at the level of mRNA accumulation.

3.2.5 Antibody raised against the carboxyl-terminal peptide of *cdc2TaB* detects wheat p34^{cdc2} protein

Antibodies raised against a perfectly conserved 16 amino acid peptide, EGVPSTAIREISLLKE (usually called PSTAIR antibodies) have been widely used to identify putative *cdc2* homologues in a variety of species, but evidence from animal cells shows that PSTAIR antibody can also detect close variants of Cdc2 such as Cdk2. To raise specific antibodies for wheat *cdc2* protein, peptides were synthesised for use as antigens. These peptides corresponded to the carboxyl-terminus of wheat *cdc2TaA* and *cdc2TaB* were YFKDMEMVQ and ALEHEYFKDLDVSS respectively. A rabbit antiserum against the carboxyl-terminal of Cdc2TaB was generated, and called anti-cdc2TaB. Western blotting of 50 µg samples of total protein extract from wheat tissue showed that anti-cdc2TaB antibody detected a protein band migrating at about 34 kDa which was the same as the band detected by anti-PSTAIR antibody (see Fig. 3.14A). There was a dominant band (34 kDa) in wheat leaf meristem (lanes 2 and 5) and root meristem (lanes 3 and 6), while in samples containing the same amount of total extracted protein little or none of the protein was detected in the mature leaf blade (lanes 1 and 4). This result was consistent with earlier observations that high level of p34^{cdc2}-like protein was restricted to regions of cell proliferation in seedling wheat leaf (John et al., 1990). In controls performed by immunoblotting total cell lysates of yeast *S. cerevisiae* in parallel with wheat meristem extract, anti-cdc2TaB antibody could detect a 34 kDa band only in wheat meristem extract but not in yeast extract; while anti-PSTAIR antibody could detect a 34 kDa band in both wheat and yeast extract (Fig. 3.14B). The antibody against the carboxyl-terminal of Cdc2TaB is therefore specific for wheat Cdc2-like protein. When Cdc2TaA or Cdc2TaB protein was expressed in yeast *cdc28^{ts}* mutants (see Chapter 4), the total cell lysates of yeast mutants were immunoblotted by anti-cdc2TaB antibody and the antibody could detect both Cdc2TaA and Cdc2TaB protein (Fig. 4.9). Therefore the anti-cdc2TaB antibody shows reaction with both wheat *cdc2* proteins, probably because in the 14 amino acids of the Cdc2TaB carboxyl-terminal region, which was chosen for raising antibody, there are 9 amino acids in common with

the Cdc2TaA carboxyl-terminal (Fig. 3.11A). A more extreme terminal region (a smaller peptide) with smaller overlap between the two sequences has not yet proven antigenic. For example, the carboxyl-terminal 9 amino acids of Cdc2TaA was tested and found unsuccessful. A longer peptide equivalent to the carboxyl-terminal of Cdc2TaA has been synthesised and the generation of rabbit antisera is now under way. It will be interesting to test any antibody that results for its ability to discriminate between the two Cdc2-like proteins. If this antibody strategy fails, the examination of possible differential expression of the types A and B *cdc2*-like genes of wheat may be possible by identification of regions in which the nucleotide sequence may be sufficiently different for a probe based on that region, when used at high stringency, to bind preferentially to a single species of mRNA. A possible region is the 3' non-translated region where nucleotide difference is quite different between two genes (only about 20% identity, see Fig. 3.4 and Fig. 3.5). If this is impossible it may be necessary to clone the genomic gene and couple its promoter to a reporter gene; however this strategy requires that the construct with reporter is reintroduced into the genome of a wheat plant and the present state of cereal transformation technology makes this difficult to accomplish quickly.

3.3 Discussion

3.3.1 Relationship of *cdc2TaA* and *cdc2TaB*

Based on sequence similarity I have identified two wheat *cdc2*-like genes, *cdc2TaA* and *cdc2TaB*, both encoding proteins that are structurally very similar to the $p34^{cdc2}$ kinase of *S. pombe* (*cdc2*), *S. cerevisiae* (*CDC28*), human (*cdc2Hs*) and other plant *cdc2* homologues (Hindley and Phear, 1984; Lorincz and Reed, 1984; Lee and Nurse, 1987; Fereira et al., 1991; Hirt et al., 1991, 1993; Colasanti et al., 1991; Hashimoto et al., 1992). Both wheat *cdc2*-like genes have about 60% identity of amino acid sequence with yeast and animal *cdc2* genes and 82-92.5% identity of amino acid sequence with plant *cdc2* homologues (see Table 3.2 and Fig. 3.12). The high degree of homology

among plant *cdc2* genes, especially among wheat, rice and maize *cdc2* reveals the close evolutionary relationship between the cereal plants.

That the cloned genes are translated into wheat protein is indicated by the ability of antibody raised against the C-terminal region of *cdc2TaB* to detect a band of 34 kDa on Western blot of wheat meristem protein. This 34 kDa band is also detected by antibody against the 16 amino acid "PSTAIR" domain of *cdc2* protein (see Fig. 3.14). This evidence together with the observation that both protein and mRNA of the genes is preferentially expressed in meristem regions indicates that *cdc2TaA* and *cdc2TaB* have the properties expected of wheat *cdc2*-like genes.

The relationship between *cdc2TaA* and *cdc2TaB* is of interest. Since wheat is a hexaploid organism, the possibility exists that the two *cdc2*-like genes might be alleles, however the considerable difference between *cdc2TaA* and *cdc2TaB*, which have only 79.7% identity at the nucleotide level and 85% identity at the amino acid level, indicates they are different genes. Alternatively, the two wheat *cdc2*-like genes might be homoeologues, that are equivalent genes derived from separate genomes that contribute to modern hexaploid wheat. In this case, it may follow that one of the *cdc2* genes in one of the three genomes is silenced. Rice is a diploid and possesses a pair of *cdc2*-like genes. It is more likely that each of the three wheat progenitors (genomes) also carries a pair of *cdc2*-like genes and in 2n cells of hexaploid wheat, they constitute a pair of homoeologous loci. The variant RACE products A1 and A1* (see 3.2.2) might present the homoeologues of the type A *cdc2*-like gene. The possibility that genes are homoeologues can be verified by Southern blot analysis. However, my experiments with wheat genomic DNA Southern hybridisation, using *cdc2TaA* cDNA as a probe, were not successful. The failure was probably due to the large size of the wheat genome (4 times bigger than maize and 196

times bigger than *Arabidopsis*), together with likely presence of the *cdc2* gene as a single copy per genome, as it has been found to be in rice, alfalfa and *Arabidopsis* (Hashimoto et al., 1992; Hirt et al., 1993; Ferreira et al., 1991). I have not had time to attempt improvements to the experimental conditions to obtain successful Southern hybridisation.

In yeast, $p34^{cdc2}$ is essential for progression of the cell cycle at both the G1-S and G2-M transitions (Nurse and Bissett, 1981) by association with G1 cyclins or G2 cyclins respectively (Booher and Beach, 1987, 1988; Hadwiger et al., 1989b; Solomon et al., 1990). In higher eukaryotes, multiple *cdc2*-like genes have been found in animal and plant cells (Lehner and O'Farrell, 1991; Elledge and Spottswood, 1991; Ninomiya-Tsuiji et al., 1991; Paris et al., 1991; Meyerson et al., 1992; Hirayama et al., 1991; Colasanti et al., 1991; Hashimoto et al., 1992; Hirt et al., 1993; Miao et al., 1993). Some of these clearly have distinct functions from *cdc2* and the best characterised of these is the cyclin-dependent kinase 2 (*cdk2*) gene product, $p33^{cdk2}$, a close relative of $p34^{cdc2}$, that is found in animal cells. $p33^{cdk2}$ binds preferentially to Cyclin A and E and is active at the G1-S phase transition rather than at mitosis (Pines and Hunter, 1990; Fang and Newport, 1991; Pagano et al, 1992; Rosenblatt et al., 1992; Koff et al., 1992; Dulic et al., 1992). In plants, almost every investigated species has revealed two relatively similar *cdc2*-like genes, for example in *Arabidopsis*, alfalfa, soybean, rice and maize (as documented above). So it is not surprising that I have detected two *cdc2*-like genes in wheat. However it remains to be established whether any *cdk2* gene has been detected in plants.

To address the question of whether either of the wheat *cdc2*-like genes might be identified as a putative *cdk2*, I made a comparison between known *cdc2* and *cdk2* genes selecting *cdk2* sequences from *Xenopus*, human and goldfish as being taxonimically diverse vertebrates (see Fig. 3.12). There are 23 conserved amino acids common to these *cdk2* genes which differ from all *cdc2* genes. These difference regions, where Cdk2 structure diverges from Cdc2, may include regions where the structure of Cdk2 is specialised for performing Cdk2-specific functions, such as the phosphorylation of proteins concerned with the transcription of genes involved in DNA synthesis (Lees et al., 1992; Hinds et al., 1992). Certainly the conserved points of difference in Cdk2 provide one possible means to distinguish Cdk2 from Cdc2. Comparison of these places

in wheat Cdc2TaA and Cdc2TaB genes with Cdk2 showed in all 23 cases that both *cdc2TaA* and *cdc2TaB* are not *cdk2*-like (Fig. 3.12).

Since *cdc2TaA* could complement budding yeast *cdc28*^{ts} mutants, *cdc28-13*^{ts} and *cdc28-1N*^{ts} (see Chapter 4), *cdc2TaA* is a *cdc2*-like gene of wheat. However in the same tests *cdc2TaB* could not complement, I therefore compared the protein sequences of Cdc2TaA and Cdc2TaB to search for non-*cdc2* elements in Cdc2TaB and found there were 43 amino acids different (see Fig. 3.11A). In 24 cases the substitution was of an amino acid of the same functional type. In the 19 cases where an amino acid of different functional type was substituted in *cdc2TaB*, there was nonetheless in 14 of these instances an amino acid in another plant *cdc2* gene that was of the same functional type as in *cdc2TaB*. Only 5 substitutions in the whole molecule introduced an amino acid of functional type not present in any known plant *cdc2* at that position (using functional homologues of plant *cdc2* genes shown in Fig. 3.12). These substitutions were not clustered in any part of the molecule, but located at less conserved parts of *cdc2* molecules. The small difference of amino acid sequence in Cdc2TaB compared with Cdc2TaA does not point strongly to a separate function. By contrast the sequence of Cdk2, which does differ in function from Cdc2 in human, has only 65% sequence identity (Elledge and Spottswood, 1991; Ninomiya-Tsuji et al., 1991)

The crystal structure of human Cdk2 (De Bondt et al., 1993) and Cdk2-cyclinA complex (Jeffrey et al., 1995) has revealed that the activation of Cdk2 kinase by cyclin A binding is due to conformational change of Cdk2 that allows the catalytic cleft to bind substrates. The critical Cdk2 regions involved in binding to cyclin A are around the T-loop, at amino acids 145-170, as well as the PSTAIR region. Since animal p34*cdc2* is functional in complex with cyclin B while Cdk2 is active in complex with cyclin E and A, differences in the cyclin binding region might be characteristic. However examination of these critical regions in Cdk2 and Cdc2 homologues, showed the amino acids were almost all conserved in both Cdc2 and Cdk2 proteins (Fig. 3.12), thus it is hard to tell the difference between animal Cdc2 and Cdk2 based on their structure in these regions, therefore the identity of plant type B genes, which do not share identifiable common structural themes, cannot be established from their sequence in cyclin binding regions.

To pursue further the possibility that the pairs of *cdc2*-like genes found in other plants might contain one *cdc2*-like and one *cdk2*-like, I examined the best characterised, which were isolated from alfalfa by Hirt et al. (1993). The two alfalfa *cdc2*-like genes might encode proteins with different cell cycle functions since *cdc2MsA* was able to complement fission yeast *cdc2-33*^{ts} and budding yeast *cdc28-1N*^{ts} which are blocked in G2 phase at the restrictive temperature; in contrast, *cdc2MsB* was only able to complement budding yeast *cdc28-4*^{ts} which is blocked in G1 phase at the restrictive temperature, but not able to complement fission yeast *cdc2-33*^{ts} and budding yeast *cdc28-1N*^{ts} (Hirt et al., 1993). The possibility was considered that wheat type B, which cannot complement *cdc28*^{ts} alleles as will be described in Chapter 4, might be equivalent to alfalfa *cdc2MsB*. The two genes might perhaps identify a plant-specific *cdk2*, which could be distinct from *cdc2* and from animal *cdk2*. To test this possibility, amino acid sequences were examined to determine whether the pair of *cdc2*-like genes in wheat differed from each other in the same places as the pair of *cdc2*-like genes in alfalfa differed from each other (this comparison is shown in Fig. 3.11). There are 43 places where the two wheat genes differed from each other but the alfalfa pair shared only 13 of them and in 6 of these places the amino acid in MsB differed from that in TaB, the 7/13 (54%) similarity of MsB with TaB at the points where they both shared differences from the other *cdc2*-like gene in each plant, is lower than the overall similarity of 85% between MsB and TaB. There is therefore no evidence that regions of difference between MsA and MsB and between TaA and TaB are regions where the B-types are specialised to perform functions in common, and therefore no evidence that they are plant equivalents of *cdk2*. Rather random drifting apparent of duplicate genes in both alfalfa and wheat (perhaps each from separate progenitors of the hexaploid wheat) is a more likely explanation of the pairs of *cdc2*-like genes.

There is therefore no evidence from sequence comparison that wheat *cdc2TaB* has sequence similarity with *cdk2* or with alfalfa type B *cdc2* gene. Consequently it is unlikely that type B has a specific function in a particular part of the cell cycle in the way that *cdk2* does, although there is evidence in fission yeast that low activity of p34^{cdc2} is needed at G1-S (Hayles et al., 1994) and TaB protein could provide this. In yeasts the

single p34^{cdc2} molecule performs the G1-S as well as the G2-M functions therefore the G1-S functions need not require numerous specific sequences, and plants could be intermediate between yeasts and animals in having a G1-S kinase only subtly different from the G2-M kinase.

It is also appropriate to consider what alternative possible cause may have resulted in the evolutionary persistence in wheat of the two distinct *cdc2* genes. A possibility is that they have different promoter regions that are responsive to different hormones and therefore allow the different hormone combinations that specify cell proliferation in different regions of the plant to be able to induce expression of at least one *cdc2* gene. The basis of this suggestion is that root and shoot tissues in general have different hormone requirements. In the root a higher ratio of auxin to cytokinin is optimum for cell proliferation than is the case in the shoot. In the root, raised cytokinin can signal cessation of division but in the shoot induces initiation of division (Torrey 1956; Hamill 1993). Interaction of plant hormones with *cdc2* expression is indicated by the inducing effects of auxin on *cdc2* mRNA levels, which for example are elevated in pea root tissue within 10 min (John et al., 1993b). In conformity with the hypothesis of differential expression in root and shoot, there is evidence in soybean of two *cdc2*-like genes that are expressed differentially in root and shoot, both of which genes can complement *cdc28^{ts}* (Miao et al., 1993). In monocotyledonous tissues a different spectrum of hormones may be involved, although the auxin analogue 2,4-D was effective in supporting continued division in leaf meristem (Chapter 5). Particular morphological responses may well involve response to particular hormones. In deepwater rice, gibberellin (GA) can promote rapid internodal growth by increasing the transcriptional levels of the *cdc2* and cyclin genes (*cdc2Os2*, *cycOs1* and *cycOs2*), and then enhancing the activity of a histone H1 kinase which may be p34^{cdc2} although it has not been characterised by p13^{suc1} or antibody binding. The induction of *cdc2* mRNA level by GA treatment in rice internode acts only on *cdc2Os-2*, but not *cdc2Os-1*. The change in rice *cdc2* mRNA level was followed by reverse transcriptional PCR of two specific cDNA sequences at the 3' noncoding region to quantify products from the two different genes, which were

expressed differentially (Sauter et al., 1995). The independent expression of two rice *cdc2* genes is consistent with response to different hormone ratios.

To investigate whether the pair of wheat cdc2-like genes is similar to that of soybean or to that of rice, I did a sequence comparison between the three pairs. Wheat Cdc2TaA is 84.1% identical to soybean Cdc2-S5 and 85.8% to Cdc2-S6; Cdc2TaB is 85.1% identical to Cdc2-S5 and 85.8% to Cdc2-S6. In 43 places where the two wheat genes differed from each other, the soybean pair were different in only 7 of them and in 6 of these places the amino acid in soybean differed from that in TaB (Fig. 3.11). It is therefore apparent that the pair of wheat *cdc2*-like genes is not similar to the pair of soybean *cdc2*-like genes.

The pair of wheat genes is however very similar to the rice pair. Wheat Cdc2TaA is similar to rice Cdc2Os1 with 92.5% identity, while Cdc2TaB is similar to Cdc2Os2 with 91.8% identity (Table 3.2). In 43 places where the two wheat genes differed from each other, the rice pair were also different in 30 of them and in 22 of these places the amino acid in rice Cdc2Os2 is the same as that in wheat Cdc2TaB (Fig. 3.11). This similarity indicates that rice and wheat are closely related species and may in turn provide an indication that rice type 2 and wheat type B have similar functions and could both be expressed in response to an ancillary hormone which in rice is GA. It is significant that only strains of wheat that are responsive to GA can grow tall (Bush and Evans, 1988). It could therefore be that in both cereals stimulation of internode elongation by cell division occurs by GA mediated induction of one of the *cdc2* genes.

3.3.2 Wheat *cdc2*-like genes are transcriptionally regulated in development

Northern analysis carried out on various tissues indicated that expression of wheat *cdc2* genes correlated with cell proliferation. In proliferating yeast cells (Durkacz et al., 1986) and in cultured mammalian cells (McGowan et al., 1990), *cdc2* mRNA does not vary through the cell cycle, however during the course of development animal cells do employ regulation of *cdc2* mRNA level (Krek and Nigg, 1989; Lee et al., 1988). The evidence from Northern blot analysis of alfalfa (Hirt et al., 1993), maize (Colasanti et al.,

1991), rice (Hashimoto et al., 1992), petunia (Bergounioux et al., 1992), soybean (Miao et al., 1993), and *in situ* hybridisations in *Arabidopsis* (Martinez et al., 1992), show that a high level of *cdc2* mRNA is detectable at meristems, in proliferative suspension cells and in young organs during the period of cell division. In higher eukaryotes short-term changes in *cdc2* kinase activity (eg. during cell cycle progression) may be mediated primarily by post-translational modifications including phosphorylation and formation of complexes with stimulating proteins such as cyclins and inhibitory proteins such as CKIs, whereas long-term changes such as during cell differentiation might involve controls operating at the level of *cdc2* gene expression (Lee et al., 1988). The first evidence that this is the case in plant development came from the distribution of p34^{cdc2} in seedling wheat leaf (John et al., 1990). The successful cloning of wheat *cdc2* genes opened the way to determine whether change in mRNA level might contribute to determining the change in protein level during the switch to cell differentiation.

I chose for Northern analysis (1) wheat leaf material 0-10 mm from the leaf base in the meristem, (2) 30-40 mm from the base where cells are undergoing differentiation and only small amounts of p34^{cdc2}-like protein could be detected, (3) 80-90 mm from the base where cells are differentiated and very little p34^{cdc2}-like protein could be detected (John et al., 1990; and see Chapter 5). The Northern hybridisation showed that high *cdc2* mRNA was detected in the leaf meristem but little or no *cdc2* mRNA was detectable in the middle or tip region of leaf even in long exposures of the Northern blot. A similar result has been obtained in maize leaf where mature differentiated cells showed a very low level of *cdc2*-hybridising mRNA but in maize a low signal was obtained from differentiated cells by longer exposure, suggesting that transcription of *cdc2* gene does not cease completely in these terminally differentiated cells (Colasanti et al., 1991).

In *Arabidopsis*, Hemerly et al. (1993) have suggested that expression of the *cdc2a* gene is linked with competence for cell division. *Arabidopsis cdc2a* can be transcriptionally regulated by plant-mitogenic signals, such as hormones, light and wounding and is correlated with proliferative competence, eg. in root pericycle cells. Expression is not always coupled with proliferation but always precedes it (Hemerly et al., 1993). Thus *cdc2* gene expression is necessary but not itself sufficient, indicating

that other positive signals are required. One such class of signal is likely to be the presence of cyclin proteins, with which p34^{cdc2} must complex to be enzymically active. In *Arabidopsis* the cyclin gene *cyc1At* is expressed almost exclusively in dividing cells, and induction of *cyc1At* expression is a very early event during lateral root formation induced by auxin. Also in tobacco protoplasts which were transformed with a *cyc1At* promoter-*gus* gene construct, an increase in *cyc1At* expression was observed only when cell division was induced by phytohormone treatment (Ferreira et al., 1994b), suggesting that *Cyc1At* accumulation in *Arabidopsis* is transcriptionally regulated and might be one of the limiting factors for the activation of cell division. It is possible that expression of the *cdc2* gene confers the potential for cell division, while expression of cyclin genes and we may assume other *cdc2* regulating proteins, will produce the necessary binding proteins and phosphorylation-controlling enzymes to activate p34^{cdc2} kinase and drive cell division.

A major difference between plant cells and animal cells is that most cells of the dicotyledonous group of plants can, under appropriate conditions, re-enter the cell cycle and develop into whole plants, a characteristic known as totipotency. Accordingly, Hemerly and his colleagues (1993) proposed a model in which dividing cells and competent cells contain high levels of *cdc2a* mRNA; differentiating cells decrease *cdc2a* mRNA levels; terminally differentiated cells have no *cdc2a* expression and differentiated cells that retain totipotency, have low levels of *cdc2a* mRNA. In differentiated cells induction of *cdc2a* expression by wounding or seasonally induced change in hormone level, might precede or induce competence for cell division by resulting in increased levels of *cdc2a* mRNA. Subsequent activation of p34^{cdc2} might depend on further signals (eg. hormone and light) impelling competent cells to division (Hemerly et al., 1993). My observation that *cdc2* mRNA is not detectable in differentiating and differentiated wheat leaf tissues correlates with their zero potential for totipotency. In rice, mature leaf tissue also contains no detectable *cdc2* mRNA (Hashimoto et al., 1992). A common phenomenon in monocotyledonous plants is that mature cells can hardly resume division either in normal plant development or in artificial culture with physiological level of supplied auxin (Wernicle et al., 1986). Wheat leaf cells have

conformed with this pattern and differentiating and differentiated cells have proved to be not capable of resuming division (see Chapter 5) although media were employed that are capable of stimulating continued division in cells that had not yet differentiated. It is therefore plausible that absence of *cdc2* mRNA is in cereals a molecular marker for the irreversible loss of competence for cell division.

3.3.3 GC-rich leader sequence in transcripts of both wheat *cdc2*-like genes; possible translational regulation

Both wheat *cdc2*-like cDNAs showed regions of high GC content in 5' noncoding sequences (76.3% GC in *cdc2TaA* and 88% GC in *cdc2TaB*), which implies presence of secondary structure in the molecule with the consequence that translation of these genes might be poor. In vertebrates, mRNAs with GC-rich leader sequences have been found in proteins that are expressed at low levels, such as many oncoproteins, growth factors, transcription factors, signal transduction components, and a wide variety of receptor proteins. Conversely GC rich leaders are absent from mRNAs that encode highly expressed proteins such as globins, albumins, caseins, immunoglobulins or histones (reviewed by Kozak, 1991b). It is therefore likely that GC-rich leader sequences are not redundant or random, rather their occurrence may correlate with translational regulation. According to Kozak's extensive analysis, initiation of translation in higher eukaryotes is influenced by nucleotides flanking the AUG start codon. Initiation of translation is particularly stimulated by a purine (A or G) in position 3 upstream from the A of the AUG start codon, and G in position 4 downstream from there (reviewed by Kozak 1991a). The sequence surrounding the presumed ATG start codon of two wheat *cdc2*-like cDNAs indicate that *cdc2TaB* mRNA has a sequence likely to favour translation, while *cdc2TaA* has an unfavourable context.

Furthermore, upstream of the presumed ATG of *cdc2TaA*, there is another open reading frame (ORF) which encodes a 24 amino acid polypeptide. Presence of two ORFs in a single mRNA is a rare phenomenon in higher eukaryotes, although polycistronic mRNAs are usual in prokaryotes. In eukaryotes initiation of translation

usually starts at the first AUG of a transcript and two proteins are inefficiently translated from one messenger-RNA by eukaryotic ribosomes. In some cases ribosomes can reinitiate translation at a downstream AUG codon with low efficiency when an upstream ORF is small and the distance between the two cistron is long (>79 nucleotides) (Liu et al., 1984; Kozak 1987). The separation of 108 nucleotides between the two ORFs of *cdc2TaA* mRNA means that the larger ORF is likely to be translated although with low efficiency.

In addition to reducing translation efficiency the presence of small upstream ORFs can be used by eukaryotes in translational control. For example in the yeast *S. cerevisiae*, four small upstream ORFs are present in *GCN4* mRNA and have been found to be important in translational control (Abastado et al., 1991). Also in *S. cerevisiae* the *CPA1* gene, which encodes the glutaminase enzyme, is found to have a 25 codon upstream ORF, the product of which plays an essential, negative role in the specific repression of *CPA1* by arginine (Werner et al., 1987).

Therefore the analysis of upstream sequences in *cdc2TaA* mRNA indicates that it is likely to be translated with lower efficiency than *cdc2TaB* and could be subject to translational regulation. The circumstances, in which this might occur, cannot at present be deduced but might include hormonal shifts, as in flowering, or change in level of messengers signalling environmentally induced stress, as in drought. The possibility of translational control of *cdc2* expression is not entirely without precedent since an absence of correlation between levels of *cdc2* mRNA and p34^{cdc2} has been seen in chicken development. Interestingly, chicken *cdc2* cDNA also has rich-GC (75%) sequence in the 5' noncoding region, but no upstream ORF (Krek and Nigg, 1989).

The upstream ORF may alternatively represent an incompletely processed mRNA perhaps with an unspliced intron. A number of claims of mRNAs with ATG-burdened 5' noncoding sequences have been resolved by finding that the 5' portion of the cDNA contains an intron (reviewed by Kozak, 1991b). If the *cdc2TaA* cDNA was isolated from an incompletely processed mRNA then it could be larger than the size of fully processed mRNA detected on Northen blot. That size comparison cannot be made however because the isolated *cdc2TaA* cDNA had lost an unknown number of

untranslated nucleotides and the poly(A) region from its 3' end, which reduced its size to only 1.17 kb (Fig. 3.9) and made it smaller than the 1.5 kb average size of mature *cdc2Ta* mRNA (Fig. 3.13).

Another way to assess whether the isolated cDNA is not fully processed is to search for other cDNAs of the same gene to see if they have a smaller 5' sequence that might be the result of more complete processing. Interestingly, two other isolates from the library were significantly larger molecules of 1.5 kb. In spite of this large size they were incomplete at the 3' end, having only the first 500 bases of the *cdc2* ORF and a 5' region of 1 kb. This apparent large size in the 5' untranslated region is difficult to reconcile with an average size of 1.5 kb of the intact mRNA seen on Northern blot (Fig. 3.13A). Fully processed mRNA of 1.5 kb is likely to comprise 882 base ORF (Fig. 3.9) with at least 206 base 3' untranslated sequence (Fig. 3.4) and therefore the maximum size in the 5' untranslated region is 412 bases. This 412 base size deduced for the 5' untranslated region of the most abundent size of *cdc2TaA* mRNA seen on Northern blot is smaller than the 1 kb of 5' untranslated cDNA seen in larger cDNA isolates. Unfortunately the identity of the 1 kb of 5' untranslated cDNA in the two 1.5 kb cDNA isolates could not be determined, because sequencing in this untranslated region was unsuccessful. Although a 3' to 5' primer binding in the *cdc2* ORF was able to show the N-terminal portion of the *cdc2* gene, it could not prime synthesis further into the 5' untranslated region. It may be that the 5' untranslated region had excessive secondary structure. It is not clear that the GC-rich sequences in *cdc2TaA* and *cdc2TaB* and the upstream ORF in *cdc2TaA* are the results from incompletely processed mRNAs or not.

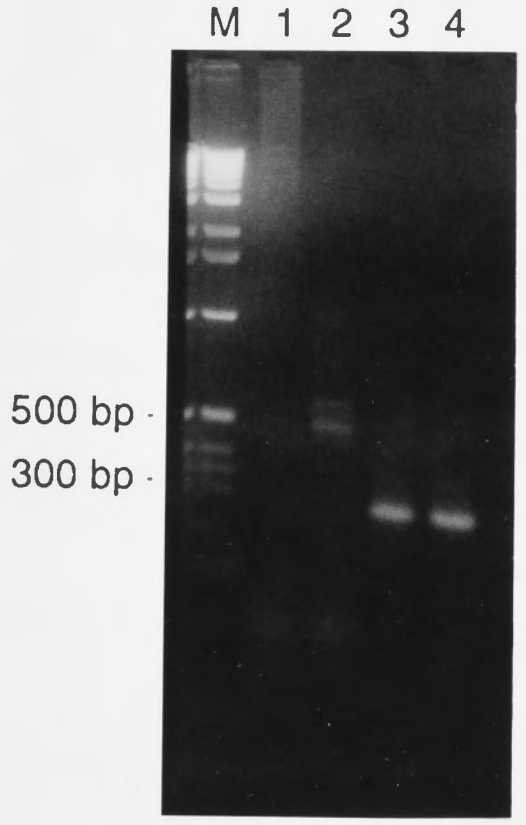


Fig. 3.1 RT-PCR with wheat mRNA template (lane 1, 2) and subsequent nested PCR using the product of the first amplification as template, yielded a 280 base pair product that corresponded to the expected size for a *cdc2* coding region between primers 5'cdc2/II and 3'cdc2/I.

1, After first-strand cDNA was synthesised by using oligo$(dT)_{15}$ as primer in the reverse transcriptase reaction, primers 5'cdc2/I and 3'cdc2/II and Taq DNA polymerase were added to the reaction and thermocycled, at 94°C 1 min, 55°C 2 min, 72°C 3 min, for 30 cycles. 5 µl from 50 µl reaction was loaded on a 2% agarose gel (lane 1).

2, First-strand cDNA was synthesised using 3'cdc2/II as the primer, 5'cdc2/I and Taq DNA polymerase was then added to the reverse transcriptase reaction and thermocycled as described above. 5 µl from 50 µl reaction was loaded on the gel (lane 2).

3, Nested PCR using as template the reaction products seen in lane 1, with internal primers 5'cdc2/II and 3'cdc2/I and 1/25 volume of the first reaction as the template, thermocycled, at 94°C 1 min, 55°C 1 min, 72°C 2 min, for 25 cycles. 5 µl from 50 µl reaction was loaded on the gel (lane 3).

4, Nested PCR using as template the reaction products seen in lane 2, with internal primers 5'cdc2/II and 3'cdc2/I and 1/25 volume of the first reaction as the template, thermocycled as described in **3**. 5 µl from 50 µl reaction was loaded on the gel (lane 4).

Lane M contains 1 kb DNA ladder (GIBCO BRL).

Type A:

```
          ********** ********** ****
    1  GAGGGCGTCC CCTCCACCGC CATCCGCGAG ATCTCGCTCC TCAAGGAGAT    50
       E  G  V  P   S  T  A   I  R  E   I  S  L  L   K  E  M

   51  GCAGCACGGC AACATCGTCA AGCTGCACGA TGTTGTCCAC AGCGAGAAGC   100
        Q  H  G   N  I  V  K   L  H  D   V  V  H   S  E  K  R

  101  GCATATGGCT CGTCTTTGAG TACCTGGATC TGGACCTGAA GAAGTTCATG   150
         I  W  L   V  F  E   Y  L  D  L   D  L  K   K  F  M

  151  GACTCCTGTC CAGAGTTTGC CAAGAGCCCC GCCTTGATCA AGTCATATCT   200
       D  S  C  P   E  F  A   K  S  P   A  L  I  K   S  Y  L

                                                            *
  201  CTATCAGATA CTCCGCGGCG TTGCTTACTG TCATTCTCAT AGAGTTCTTC   250
        Y  Q  I   L  R  G  V   A  Y  C   H  S  H   R  V  L  H

          ********** ********** ***
  251  ATCGAGATTT GAAACCTCAG AAT  273
         R  D  L   K  P  Q  N
```

Type B:

```
          ********** ********** ****
    1  GAGGGCGTCC CGTCCACCGC CATCCGCGAG ATCTCCCTCC TCAAGGAGAT    50
       E  G  V  P   S  T  A   I  R  E   I  S  L  L   K  E  M

   51  GCAGCACCGG AACATCGTCA GGCTGCAGGA CGTGGTGCAC AACGAGAAGT   100
        Q  H  R   N  I  V  R   L  Q  D   V  V  H   N  E  K  C

  101  GCATCTACCT CGTCTTCGAG TACCTCGACC TCGACCTCAA GAAGCACATG   150
         I  Y  L   V  F  E   Y  L  D  L   D  L  K   K  H  M

  151  GACTCCTCCT CGGACTTCAA GAACCACCAC ATAGTCAAGT CCTTCCTCTA   200
       D  S  S  S   D  F  K   N  H  H   I  V  K  S   F  L  Y

                                                        ****
  201  CCAGATCCTG CGCGGCATCG CCTACTGCCA CTCGCACCGC GTGCTTCACA   250
        Q  I  L   R  G  I  A   Y  C  H   S  H  R   V  L  H  R

          ********** **********
  251  GGGATTTCAA GCCCCAGAAT   270
         D  F  K   P  Q  N
```

Fig. 3.2 Nucleotide sequence and deduced amino acid sequence of two 280 bp wheat *cdc2*-like gene fragments, amplified by reverse transcription of wheat total RNA followed by polymerase chain reaction using oligonucleotides 5'cdc2/II and 3'cdc2/I (marked with *). The underlined oligonucleotides are the regions that were selected as different in type A and B and suitable for synthesis as primers (5'cdc2A and 5'cdc2B) for rapid amplification of 3' end of wheat *cdc2* cDNAs.

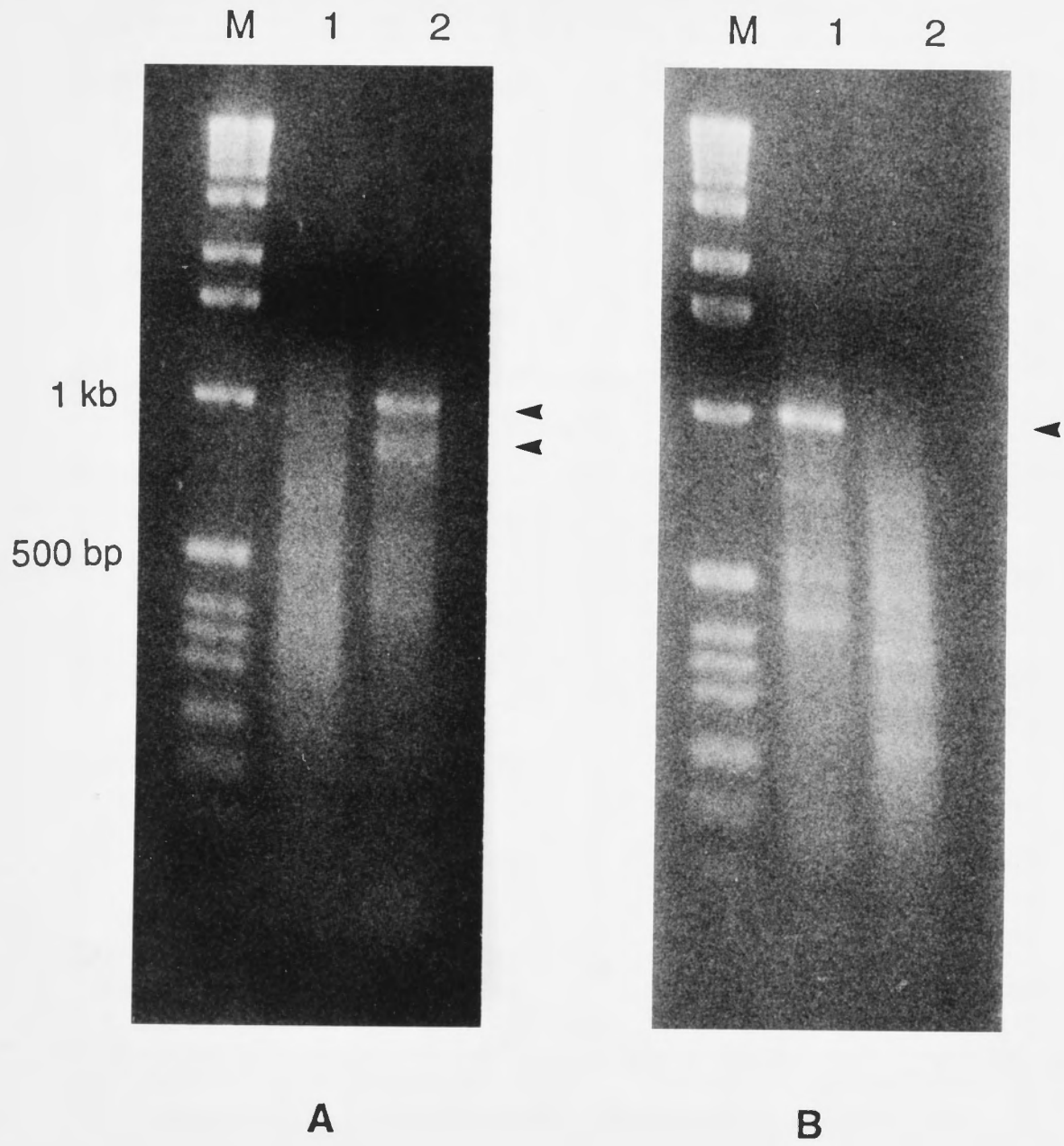


Fig. 3.3 Rapid amplification of 3' end of wheat *cdc2*-like cDNA. First-strand cDNA was synthesised as in RT-PCR and then used as template in the following PCR:

A, Initial PCR with primers oligo$(dT)_{17}$-RI and 5'cdc2/II, shown in lane 1; then nested PCR with primers oligo$(dT)_{17}$-RI and 5'cdc2A, shown in lane 2, yielding a 1 kb and a 800 bp band.

B, Initial PCR with primers oligo$(dT)_{17}$-RI and 5'cdc2/II, shown in lane 2; then nested PCR with primers oligo$(dT)_{17}$-RI and 5'cdc2B, shown in lane 1, yielding a 1 kb band.

M is 1 kb DNA ladder (GIBCO BRL).

```
  1  TTTGCCAAGAGCCCCGCCTTGGTCAAGTCATATCTCTATCAGATACTCCG   50
  1  F  A  K  S  P  A  L  V  K  S  Y  L  Y  Q  I  L  R   17

 51  CGGCGTTGCTTACTGTCATTCTCATAGAGTTCTTCATCGAGATTTGAAAC  100
 17   G  V  A  Y  C  H  S  H  R  V  L  H  R  D  L  K  P   33

101  CTCAGAATTTATTGATAGACCGGCGTACTAATGCACTGAAGCTTGCAGAC  150
 34    Q  N  L  L  I  D  R  R  T  N  A  L  K  L  A  D    50

151  TTTGGTTTAGCAAGGGCATTTGGAATTCCTGTCCGTACATTTACTCATGA  200
 51  F  G  L  A  R  A  F  G  I  P  V  R  T  F  T  H  E   67

201  GGTAGTAACATTATGGTACAGAGCTCCTGAAATCCTTCTTGGAGCAAGGC  250
 67   V  V  T  L  W  Y  R  A  P  E  I  L  L  G  A  R  Q   83

251  AGTATTCCACACCAGTTGACGTGTGGTCAGTGGGCTGTATCTTTGCAGAA  300
 84    Y  S  T  P  V  D  V  W  S  V  G  C  I  F  A  E    100

301  ATGGTGAACCAGAAACCACTGTTCCCTGGCGATTCTGAGATTGATGAGCT  350
101  M  V  N  Q  K  P  L  F  P  G  D  S  E  I  D  E  L   117

351  ATTTAAGATATTCAGGGTACTCGGCACTCCAAATGAACAAACTTGGCCAG  400
117  F  K  I  F  R  V  L  G  T  P  N  E  Q  T  W  P  G   133

401  GCGTGAGTTCCTTGCCTGACTACAAGTCCGCCTTCCCCAGGTGGCAGGCA  450
134   V  S  S  L  P  D  Y  K  S  A  F  P  R  W  Q  A    150

451  GAGGACCTTGCAACCGTTGTCCCCAATCTTGAACCTGTTGGCCTGGACCT  500
151  E  D  L  A  T  V  V  P  N  L  E  P  V  G  L  D  L   167

501  TCTCTCGAAAATGCTTCGGTTCGAGCCAAACAAGAGGATCACGGCTAGGC  550
167   L  S  K  M  L  R  F  E  P  N  K  R  I  T  A  R  Q   183

551  AGGCTCTTGAGCATGAGTACTTCAAGGACATGGAGATGGTACAGTGAGCT  600
184    A  L  E  H  E  Y  F  K  D  M  E  M  V  Q  *       200

601  GGCTATGTGGTAGTGACTGGCATATGTATGAGCTGAGCTGCTCGTTTCAT  650

651  TCCTTTTGTGAACGCTCTGCCTTCCCCTTTTCGGCATTTTTGTCTGTCAA  700

701  TTGAATATTTCAGATCTGGTGTGTTTGAGGTGCACTCAGGATTGCTACTA  750

751  AATAGATTACCATCTTGGTCTCCATTTTGTTCAACGTAAAAAAAAAAAAA  800
```

Fig. 3.4 DNA sequence and deduced amino acid sequence of the 3' end of wheat *cdc2A* cDNA (RACE-A1).

```
   1  GACTTCAAGAACCACCACATAGTCAAGTCCTTCCTCTACCAGATCCTGCT   50
   1  D  F  K  N  H  H  I  V  K  S  F  L  Y  Q  I  L  L   17

  51  CGGCATCGCCTACTGCCACTCGCACCGCGTGCTTCACAGGGATCTCAAGC  100
  17   G  I  A  Y  C  H  S  H  R  V  L  H  R  D  L  K  P   33

 101  CCCAGAACCTGCTGATTGATCGCCGCACCAATTCATTGAAGCTTGCTGAC  150
  34    Q  N  L  L  I  D  R  R  T  N  S  L  K  L  A  D    50

 151  TTCGGGTTGGCCAGGGCGTTCGGCATTCCTGTCCGGACATTTACTCACGA  200
  51  F  G  L  A  R  A  F  G  I  P  V  R  T  F  T  H  E   67

 201  GGTGGTGACATTATGGTACAGAGCACCAGAAATTCTTCTGAGTGCAAGGC  250
  67   V  V  T  L  W  Y  R  A  P  E  I  L  L  S  A  R  Q   83

 251  AGTATTCTACCCCTGTTGATGTGTGGTCGGTTGGTTGCATTTTCGCCGAA  300
  84    Y  S  T  P  V  D  V  W  S  V  G  C  I  F  A  E    100

 301  ATGGTGAATCAGAAACCCCTATTTCCTGGTGATTCTGAGATTGATGAACT  350
 101  M  V  N  Q  K  P  L  F  P  G  D  S  E  I  D  E  L   117

 351  CTTCAAGATTTTCAGAATTATGGGCACTCCTAATGAAGAAACCTGGCCAG  400
 117   F  K  I  F  R  I  M  G  T  P  N  E  E  T  W  P  G   133

 401  GTGTTTCTTCGTTACCTGACTACAAATCAGCTTTCCCCAAGTGGCCGTCC  450
 134    V  S  S  L  P  D  Y  K  S  A  F  P  K  W  P  S    150

 451  GTGGATCTCGCAACTGTGGTTCCAACACTCGAACCTTTGGGACTTGATCT  500
 151  V  D  L  A  T  V  V  P  T  L  E  P  L  G  L  D  L   167

 501  TCTCTCTAAAATGCTCTGCTTAGATCCAACCAGAAGAATCAACGCCCGAA  550
 167   L  S  K  M  L  C  L  D  P  T  R  R  I  N  A  R  T   183

 551  CCGCCCTCGAGCACGAGTACTTCAAGGATCTGGACGTATCCTCGTAGATC  600
 184    A  L  E  H  E  Y  F  K  D  L  D  V  S  S  *       200

 601  ACTTGCCCTGCTCCTCTGTAAATTAAGATCGCGTCGACTGATCGACAGCT  650

 651  TCCTGGATCGTGTACTCTGTGCTCCTTTCTCTCCTATTCTTTCTGACTCT  700

 701  GGCTGTGTAGAGAGAAATGAGAACGGAGGCGCCTCCTGATTCACCCTTCC  750

 751  GTGTAGTCCAAAGCCAATTGAAAAATGATCCTGATGCTCTGGGAGCTCTG  800

 801  CATTGCGATTTCGCCCGTATTTATATGGACATCGACGCCGGCGCCAGACT  850

 851  TGTTCATGTACGATGAATTTTGGAGTAGTAGCAGATAGTCAAGGGGCTGC  900

 901  AGTGTTTCATGAACTACCAACTGATGTGACAATGTTTGGTAGTGTACTAC  950

 951  TACTTGTACTTGGTAAACATCATTATCATCATGAATGGAATCAGTTGTGG  1000

1001  ACAACTAAAAAAAAAAAAAA  1020
```

Fig. 3.5 DNA sequence and deduced amino acid sequence of the 3' end of wheat *cdc2B* cDNA (RACE-B).

Fig. 3.6 The Alignment of RACE-A1 (upper sequence) with RACE-A1*(lower sequence). Codons where two sequences differ from each other are shown in bold and 4 differences occurred in the third place of a codon without changing amino acid coding. One difference caused the amino acid alteration, at position of 488, where a single letter of amino acid is indicated above the RACE-A1 and below the RACE-A1*. Another nucleotide difference occurred at position 592 where RACE-A1 (CAG) codes amino acid Q while RACE-A1* (TAG) codes stop. Lower case indicates the 3' noncoding region.

```
  1 TTTGCCAAGAGCCCCGCCTTGGTCAAGTCATATCTCTATCAGATACTCCG 50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
  1 TTTGCCAAGAGCCCCGCCTTGGTCAAGTCATATCTCTATCAGATACTCCG 50

 51 CGGCGTTGCTTACTGTCATTCTCATAGAGTTCTTCATCGAGATTTGAAAC 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 CGGCGTTGCTTACTGTCATTCTCATAGAGTTCTTCATCGAGATTTGAAAC 100

101 CTCAGAATTTATTGATAGACCGGCGTACTAATGCACTGAAGCTTGCAGAC 150
    ||||||||||||||||||||||||||||||||||||||||||||||||||
101 CTCAGAATTTATTGATAGACCGGCGTACTAATGCACTGAAGCTTGCAGAC 150

151 TTTGGTTTAGCAAGGGCATTTGGAATTCCTGTCCGTACATTTACTCATGA 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 TTTGGTTTAGCAAGGGCATTTGGAATTCCTGTCCGTACATTTACTCATGA 200

201 GGTAGTAACATTATGGTACAGAGCTCCTGAAATCCTTCTTGGAGCAAGGC 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 GGTAGTAACATTATGGTACAGAGCTCCTGAAATCCTTCTTGGAGCAAGGC 250

251 AGTATTCCACACCAGTTGACGTGTGGTCAGTGGGCTGTATCTTTGCAGAA 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||
251 AGTATTCCACACCAGTTGACGTGTGGTCAGTGGGCTGTATCTTTGCAGAA 300

301 ATGGTGAACCAGAAACCACTGTTCCCTGGCGATTCTGAGATTGATGAGCT 350
    |||||||||||||||||||||||||||||||||||||||||||||||| ||
301 ATGGTGAACCAGAAACCACTGTTCCCTGGCGATTCTGAGATTGATGAACT 350

351 ATTTAAGATATTCAGGGTACTCGGCACTCCAAATGAACAAACTTGGCCAG 400
    ||||||||||||||||||||||||||||||||||||||||||||||||||
351 ATTTAAGATATTCAGGGTACTCGGCACTCCAAATGAACAAACTTGGCCAG 400

401 GCGTGAGTTCCTTGCCTGACTACAAGTCCGCCTTCCCCAGGTGGCAGGCA 450
    ||||||| |||||||| ||||||||||||||||||||||||||||||||||
401 GCGTGAGCTCCTTGCCAGACTACAAGTCCGCCTTCCCCAGGTGGCAGGCA 450

                                  V
451 GAGGACCTTGCAACCGTTGTCCCCAATCTTGAACCTGTTGGCCTGGACCT 500
    |||||||||||||||||||||||||||||||||||| ||||||||||||||
451 GAGGACCTTGCAACCGTTGTCCCCAATCTTGAACCTCTTGGCCTGGACCT 500
                                  L

501 TCTCTCGAAAATGCTTCGGTTCGAGCCAAACAAGAGGATCACGGCTAGGC 550
    ||||||||||||||||||||||||||||||||||||||||||||||||||
501 TCTCTCGAAAATGCTTCGGTTCGAGCCAAACAAGAGGATCACGGCTAGGC 550

551 AGGCTCTTGAGCATGAGTACTTCAAGGACATGGAGATGGTACAGTGAgct 600
    |||||||||||||||||||||||||||||||||||||||| ||||||||
551 AGGCTCTTGAGCATGAGTACTTCAAGGACATGGAGATGGTGTAGtgagct 600

601 ggctatgtggtagtgactggcatatgta.....tgagctgagctgctcgt 645
     |||||||||||||||||||||||||||     ||||||||||||||||||
601 cgctatgtggtagtgactggcatatgtatgagctgagctgagctgctcgt 650

646 ttcattccttttgtgaacgctctgccttccccttttcggcatttttgtct 695
    ||||||||||||||||||||||||||||||||||||||||||||||||||
651 ttcattccttttgtgaacgctctgccttccccttttcggcatttttgtct 700

696 gtcaattgaatatttcagatctggtgtgtttgaggtgcactcaggattgc 745
    ||||| ||||||||||||||||||||||||||||||||||||||||||||
701 gtcaactgaatatttcagatctggtgtgtttgaggtgcactcaggattgc 750

746 tactaaatagattaccatcttggtctccattttgttcaacgtaaaaaaaa 795
    |||||||||||||||||||||||| |        ||   ||||||||
751 tactaaatagattaccatcttggtctcaa.....aaaaaaaaaaaaa 792

796 aaaaa 800
    |||||
793 aaaaa 797
```

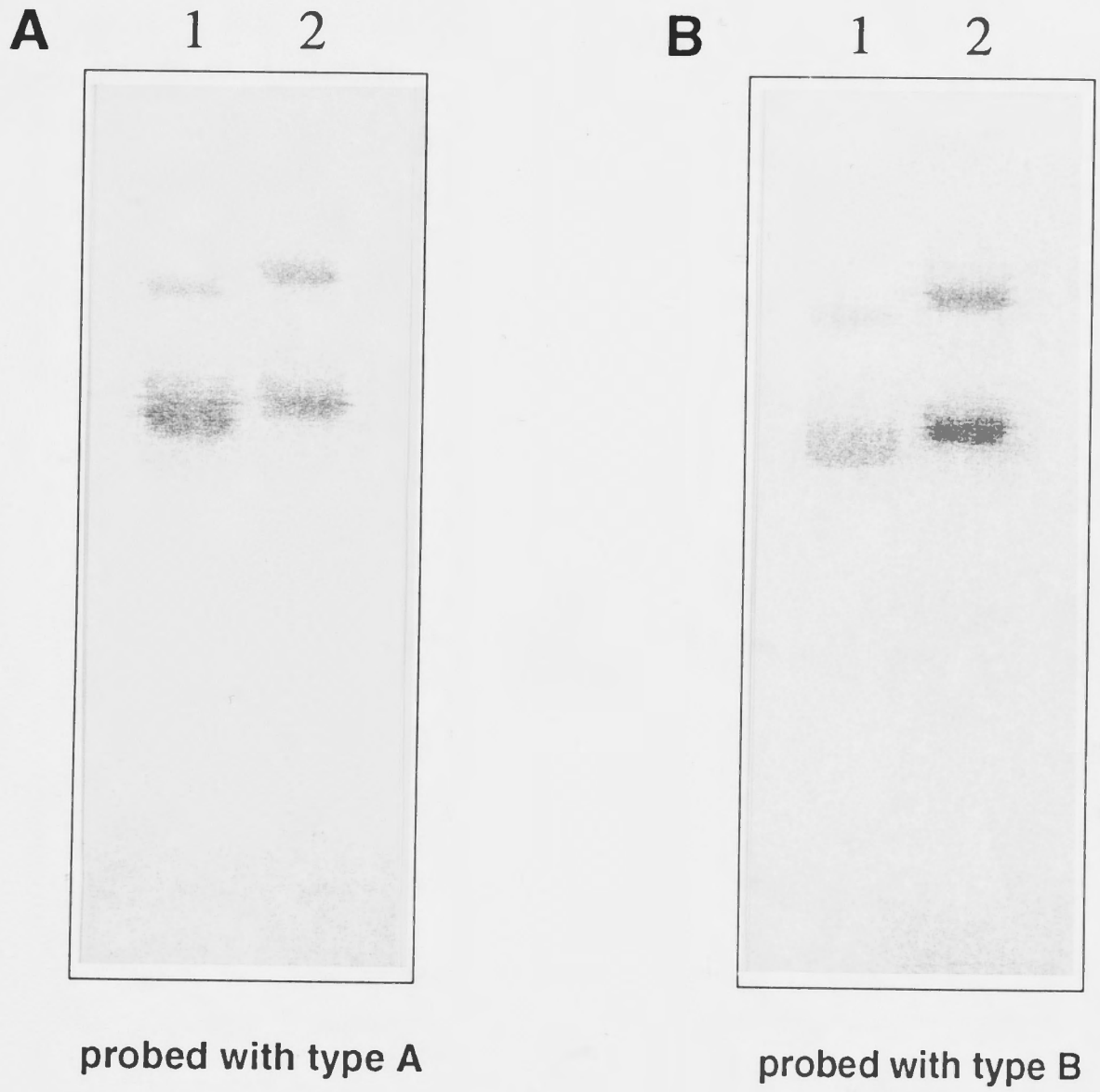


Fig. 3.7 280-bp PCR products of wheat *cdc2*-like fragments, type A and type B, can cross-hybridise with each other.

About 1 μg plasmid DNA with type A fragment (lane 1) or with type B fragment (lane 2) was loaded on 2 % agarose gel. The DNA was Southern blotted to nylon membrane and hybridised with radioactively labelled type A probe (shown in A) or type B probe (shown in B). The membranes were washed at high stringency and exposed in the PhosphorImager for 24 h.

Fig. 3.8 Isolation of positive plaques from λgt10 wheat cDNA library using the 280 bp PCR type A fragment as a probe.
A total of 2 x 10^6 phage plaques were screened and five clones, clone 4 (A), clone 5 (B), clone 9 (C), clone 10 (D) and clone 8 (picture not shown) were isolated from the sixth rescreening. The filters were probed in parallel and therefore indicate stronger hybridisation to clones 5 and 9 (B and C) than to clones 4 and 10 (A and D). Clones 5 and 9 contain *cdc2TaA* and clones 4 and 10 contain *cdc2TaB*.

```
   1  GCCCCCCTCTCCCCCTCCCCCCCACCCCCCCAATGGCGGCAGCAGCAGCA   50
  51  GCAGCAGCAGCAGCTTCGCCCGCCGCAGCCGCTCTCCCCCGCCCCTCCTC  100
 101  CCCGTGATCCCCTTCCCCTTCCCCTCCCCCGCTTCCTCCTCTCCCCCCTC  150
 151  CCGCCTCCTCACCCATTTCCCACGCCCGCGCCGCCGCCGCCGCCGTAGCA  200
 201  TTGGACGCCGACCCGATGGAGCAGTACGAGAAGGTGGAGAAGATCGGGGA  250
                    M  E  Q  Y  E  K  V  E  K  I  G  E
 251  GGGCACGTACGGGGTGGTGTACAAGGCCCGGGACAGGACCACCAACGAGA  300
       G  T  Y  G  V  V  Y  K  A  R  D  R  T  T  N  E  T
 301  CCATCGCGCTCAAGAAGATCCGCCTGGAGCAGGAGGACGAGGGCGTCCCC  350
         I  A  L  K  K  I  R  L  E  Q  E  D  E  G  V  P
 351  TCCACCGCCATCCGCGAGATCTCGCTCCTCAAGGAGATGCAGCACGGCAA  400
       S  T  A  I  R  E  I  S  L  L  K  E  M  Q  H  G  N
 401  CATCGTCAAGCTGCACGATGTTGTCCACAGCGAGAAGCGCATATGGCTCG  450
       I  V  K  L  H  D  V  V  H  S  E  K  R  I  W  L  V
 451  TCTTTGAGTACCTGGATCTGGACCTGAAGAAGTTCATGGACTCCTGTCCA  500
         F  E  Y  L  D  L  D  L  K  K  F  M  D  S  C  P
 501  GAGTTTGCCAAGAGCCCCGCCTTGATCAAGTCATATCTCTATCAGATACT  550
       E  F  A  K  S  P  A  L  I  K  S  Y  L  Y  Q  I  L
 551  CCGCGGCGTTGCTTACTGTCATTCTCATAGAGTTCTTCATCGAGATTTGA  600
       R  G  V  A  Y  C  H  S  H  R  V  L  H  R  D  L  K
 601  AACCTCAGAATTTATTGATAGACCGGCGTACTAATGCACTGAAGCTTGCA  650
         P  Q  N  L  L  I  D  R  R  T  N  A  L  K  L  A
 651  GACTTTGGTTTAGCAAGGGCATTTGGAATTCCTGTCCGTACATTTACTCA  700
       D  F  G  L  A  R  A  F  G  I  P  V  R  T  F  T  H
 701  TGAGGTAGTAACATTATGGTACAGAGCTCCTGAAATCCTTCTTGGAGCAA  750
       E  V  V  T  L  W  Y  R  A  P  E  I  L  L  G  A  R
 751  GGCAGTATTCCACACCAGTTGACGTGTGGTCAGTGGGCTGTATCTTTGCA  800
         Q  Y  S  T  P  V  D  V  W  S  V  G  C  I  F  A
 801  GAAATGGTGAACCAGAAACCACTGTTCCCTGGCGATTCTGAGATTGATGA  850
       E  M  V  N  Q  K  P  L  F  P  G  D  S  E  I  D  E
 851  GCTATTTAAGATATTCAGGGTACTCGGCACTCCAAATGAACAAACTTGGC  900
       L  F  K  I  F  R  V  L  G  T  P  N  E  Q  T  W  P
 901  CAGGCGTGAGTTCCTTGCCTGACTACAAGTCCGCCTTCCCCAGGTGGCAG  950
         G  V  S  S  L  P  D  Y  K  S  A  F  P  R  W  Q
 951  GCAGAGGACCTTGCAACCGTTGTCCCCAATCTTGAACCTGTTGGCCTGGA 1000
       A  E  D  L  A  T  V  V  P  N  L  E  P  V  G  L  D
1001  CCTTCTCTCGAAAATGCTTCGGTTCGAGCCAAACAAGAGGATCACGGCTA 1050
       L  L  S  K  M  L  R  F  E  P  N  K  R  I  T  A  R
1051  GGCAGGCTCTTGAGCATGAGTACTTCAAGGACATGGAGATGGTACAGTGA 1100
         Q  A  L  E  H  E  Y  F  K  D  M  E  M  V  Q  *
1101  GCTGGCTATGTGGTAGTGACTGGCATATGTATGAGCTGAGCTGCTCGTTT 1150
1151  CATTCCTTTTGTGAACGCTC  1170
```

Fig. 3.9 DNA sequence and deduced amino acid sequence of the wheat *cdc2TaA* gene isolated from a wheat cDNA library.

```
   1  CCCACCCCACTCCTCCCCGCCGCCGCCGCCGCCGCCCCGCTCCGATCCGC    50

  51  CCCGCGCCGCGCGGATCGCCGCGCCATGGACCAGTACGAGAAGGTGGAGA   100
                                 M  D  Q  Y  E  K  V  E  K

 101  AGATCGGGGAGGGCACGTACGGGGTGGTGTACAAGGCCAAGGACCGCTAC   150
        I  G  E  G  T  Y  G  V  V  Y  K  A  K  D  R  Y

 151  ACCAACGAGACGATCGCGCTCAAGAAGATCCGGCTGGAGCAGGAGGACGA   200
      T  N  E  T  I  A  L  K  K  I  R  L  E  Q  E  D  E

 201  GGGCGTCCCCTCCACCGCCATCCGCGAGATCTCCCTCCTCAAGGAGATGC   250
        G  V  P  S  T  A  I  R  E  I  S  L  L  K  E  M  Q

 251  AGCACCGGAACATCGTCAGGCTGCAGGACGTGGTGCACAACGAGAAGTGC   300
          H  R  N  I  V  R  L  Q  D  V  V  H  N  E  K  C

 301  ATATACCTCGTCTTCGAGTACCTCGACCTCGACCTCAAGAAGCACATGGA   350
      I  Y  L  V  F  E  Y  L  D  L  D  L  K  K  H  M  D

 351  CTCCTCCGCGGACTTCAAGAACCACCACATAGTCAAGTCCTTCCTCTACC   400
        S  S  A  D  F  K  N  H  H  I  V  K  S  F  L  Y  Q

 401  AGATCCTGCACGGCATCGCCTACTGCCACTCGCACCGTGTGCTTCACAGG   450
          I  L  H  G  I  A  Y  C  H  S  H  R  V  L  H  R

 451  GATCTCAAGCCCCAGAACCTGCTGATAGATCGCCGTACCAATTCATTGAA   500
      D  L  K  P  Q  N  L  L  I  D  R  R  T  N  S  L  K

 501  GCTTGCTGACTTCGGATTGGCGAGGGCGTTCGGCATTCCTGTCCGGACAT   550
        L  A  D  F  G  L  A  R  A  F  G  I  P  V  R  T  F

 551  TTACTCACGAGGTGGTGACATTATGGTATAGAGCACCAGAAATTCTTCTG   600
          T  H  E  V  V  T  L  W  Y  R  A  P  E  I  L  L

 601  GGTGCGAGGCAGTATTCTACCCCTGTTGATGTGTGGTCGGTTGGTTGCAT   650
      G  A  R  Q  Y  S  T  P  V  D  V  W  S  V  G  C  I

 651  TTTCGCCGAAATGGTGAATCAGAAACCTCTATTTCCTGGTGATTCTGAGA   700
        F  A  E  M  V  N  Q  K  P  L  F  P  G  D  S  E  I

 701  TTGATGAACTCTTCAAGATTTTCAGAATTATGGGCACTCCTAATGAAGAA   750
          D  E  L  F  K  I  F  R  I  M  G  T  P  N  E  E

 751  ACCTGGCCAGGTGTTTCTTCGTTACCTGACTACAAATCAGCTTTCCCCAA   800
      T  W  P  G  V  S  S  L  P  D  Y  K  S  A  F  P  K

 801  GTGGCCATCCGTGGATCTTGCAACTGTGGTTCCAACACTCGAACCTTTGG   850
        W  P  S  V  D  L  A  T  V  V  P  T  L  E  P  L  G

 851  GACTTGATCTTCTCTCTAAAATGCTCTGCTTAGATCCAACCAGAAGAATC   900
          L  D  L  L  S  K  M  L  C  L  D  P  T  R  R  I

 901  AACGCCCGAACCGCC  915
      N  A  R  T  A
```

Fig. 3.10 DNA sequence and deduced amino acid sequence of the wheat *cdc2TaB* gene isolated from a wheat cDNA library.

A) Comparison of wheat *cdc2*-like genes *cdc2TaA* and *cdc2TaB*

```
TaA     MEQYEKVEKI GEGTYGVVYK ARDRTTNETI ALKKIRLEQE DEGVPSTAIR    50
TaB     -D-------- ---------- -K--Y----- ---------- ----------    50

TaA     EISLLKEMQH GNIVKLHDVV HSEKRIWLVF EYLDLDLKKF MDSCPEFAKS   100
TaB     ---------- R---R-Q--- -N--C-Y--- ---------H ---SAD- -N    99

TaA     PALIKSYLYQ ILRGVAYCHS HRVLHRDLKP QNLLIDRRTN ALKLADFGLA   150
TaB     HHIV--F--- --H-I----- ---------- ---------- S---------   149

TaA     RAFGIPVRTF THEVVTLWYR APEILLGARQ YSTPVDVWSV GCIFAEMVNQ   200
TaB     ---------- ---------- ---------- ---------- ----------   199

TaA     KPLFPGDSEI DELFKIFRVL GTPNEQTWPG VSSLPDYKSA FPRWQAEDLA   250
TaB     ---------- --------IM -----E---- ---------- --K-PSV---   249

TaA     TVVPNLEPVG LDLLSKMLRF EPNKRITARQ ALEHEYFKDM EMVQ         294
TaB     ----T---L- --------CL D-TR--N--T ---------L DVSS         293
```

B) Comparison of rice *cdc2*-like genes *cdc2Os1* and *cdc2OS2*

```
Os1     MEQYEKEEKI GEGTYGVVYR ARDKVTNETI ALKKIRLEQE DEGVPSTAIR    50
Os2     ------V--- ---------K GKHRH----- ---------- ----------    50

Os1     EISLLKEMHH GNIVRLHDVI HSEKRIYLVF EYLDLDLKKF MDSCPEFAKN   100
Os2     --------Q- R-----Q--V -K--C---------------H ---S-D- --    99

Os1     PTLIKSYLYQ ILRGVAYCHS HRVLHRDLKP QNLLIDRRTN ALKLADFGLA   150
Os2     HRIV--F--- ----I----- ---------- ---------- S---------   149

Os1     RAFGIPVRTF THEVVTLWYR APEILLGSRQ YSTPVDMWSV GCIFAEMVNQ   200
Os2     ---------- ---------- -------A-H ---------- ----------   199

Os1     KPLFPGDSEI DELFKIFRVL GTPNEQSWPG VSSLPDYKSA FPKWQAQDLA   250
Os2     ---------- -------SIM -----ET--- -A-----I-T ----PSV---   249

Os1     TIVPTLDPAG LDLLSKMLRY EPNKRITARQ ALEHEYFKDL EMVQ         294
Os2     -V-----SS- ---------L D-S---N--A ---------- -VA         292
```

C) Comparison of soybean *cdc2*-like *cdc2-S5* and *cdc2S6*

```
S5      MEQYEKVEKI GEGTYGVVYK ARDRVTNETI ALKKIRLEQE DEGVPSTAIR    50
S6      ---------- ---------- G--------- ---------- ----------    50

S5      EISLLKEMQH RNIVRLQDVV HSEKRLYLVF EYLDLDLKKH MDSSPEFVKD   100
S6      ---------- ---------- -D--S----- ---------- -------A--   100

S5      PRQVKMFLYQ ILCGIAYCHS HRVLHRDLKP QNLLIDRRTN SLKLADFGLA   150
S6      ---------- ---------- ---------- -------S-- A---------   150

S5      RAFGIPVRTF THEVVTLWYR APEILLGSRH YSTPVDVWSV GCIFAEMVNR   200
S6      ---------- ---------- ---------Q ------I--- ---------Q   200

S5      RPLFPGDSEI DELFKIFRIL GTPNEDTWPG VTSLPDFKST FPKWPSKDLA   250
S6      ---------- ---------M ---------- ---------A ----QP---K   250

S5      NVVPNLDAAG LNLLSSMLCL DPSKRITARS AVEHEYFKDI KFVP         294
S6      ------EP-- -D------Y- ---------- -L-------- ----         294
```

D) Comparison of alfalfa *cdc2*-like genes *cdc2MsA* and *cdc2MsB*

```
MsA        GENVEKI GEGTYGVVYK ARDRVTNETI ALKKIRLEQE DEGVPSTAIR    50
MsB     MEQY-K---- ---------- ----A----- ---------- ----------    50

MsA     EISLLKEMQH RNIVRLQDVV HSDKRLYLVF EYLDLDLKKH MDSSPEFIKD   100
MsB     ---------- ---------- --E------- ---------F -------A--   100

MsA     PRQVKMFLYQ MLCGIAYCHS HRVLHRDLKP QNLLIDRRTN SLKLADFGLA   150
MsB     Q--I------ I--------- ---------- -------SS- AV--------   150

MsA     RAFGIPVRTF THEVVTLWYR APEILLGSRH YSTPVDVWSV GCIFAEMANR   200
MsB     ---------- ---------- ---------- ---------- -------I-Q   200

MsA     RPLSPGDSEI DELFKIFRIL GTPNEDTWPG VTSLPDFKST FPRWPSKDLA   250
MsB     ---F------ ---------T -----E---- ---------A --K--A----   250

MsA     TVVPNLEPAG LDLLNSMLCL DPTKRITARS AVEHEYFKDI KFVP         294
MsB     -Q-------- ----S-TCR- ---R-----G -L-------- ----         294
```

Fig. 3.11 Comparison of the pair of *cdc2*-like genes from wheat (A), rice (B), soybean (C), and alfalfa (D). Amino acids are shown by the single letter code, identical amino acid residues are indicated by hyphens. Differences that introduce amino acids of different functional type are shown in bold.

```
                                ↓↓
                        ********                                ********
EG1       MENFQKVEKIGEGTYGVVYKARNR  ETGEIV ALKKIRLDTETEGVPSTAIR      50
CDK2      MENFQKVEKIGEGTYGVVYKARNK  LTGEVV ALKKIRLDTETEGVPSTAIR      50
GFCDK2    MESFQKVEKIGEGTYGVVYKAKNK  VTGETV ALKKIRLDTETEGVPSTAIR      50

TaA       MEQYEKVEKIGEGTYGVVYKARDR  TTNETI ALKKIRLEQEDEGVPSTAIR      50
TaB       MDQYEKVEKIGEGTYGVVYKAKDR  YTNETI ALKKIRLEQEDEGVPSTAIR      50
Os        MEQYEKVEKIGEGTYGVVYRARDK  VTNETI ALKKIRLEQEDEGVPSTAIR      50
Zm        MEQYEKVEKIGEGTYGVVYKALDK  ATNETI ALKKIRLEQEDEGVPSTAIR      50
Ms           GENVEKIGEGTYGVVYKARDR  VTNETI ALKKIRLEQEDEGVPSTAIR      47
Ara       MDQYEKVEKIGEGTYGVVYKARDK  VTNETI ALKKIRLEQEDEGVPSTAIR      50

Sp        MENYQKVEKIGEGTYGVVYKARHK  LSGRIV AMKKIRLEDESEGVPSTAIR      50
Sc   MSGELANYKRLEKVGEGTYGVVYKALDLRPGQGQRVVALKKIRLESEDEGVPSTAIR      57
Hs        MEDYTKIEKIGEGTYGVVYKGRHK  TTGQVV AMKKIRLESEEEGVPSTAIR      50


EG1       EISLLKELNHPNI    VKLLDVIHTEN KLYLVFEFLNQDLKKFMDRSNISGIS   100
cdk2      EISLLKELNHPNI    VKLLDVIHTEN KLYLVFEFLHQDLKKFMDASALTGIP   100
GF        EISLLKELNHPNI    VKLHDVIHTEN KLYLVFEFLHQDLKRFMDSSTVTGIS   100

TaA       EISLLKEMQHGNI    VKLHDVVHSEK RIWLVFEYLDLDLKKFMDSCPEFAKS   100
TaB       EISLLKEMQHRNI    VRLQDVVHNEK CIYLVFEYLDLDLKKHMDSSADF KN    99
Os        EISLLKEMHHGNI    VRLHDVIHSEK RIYLVFEYLDLDLKKFMDSCPEFAKN   100
Zm        EISLLKEMNHGNI    VRLHDVVHSEK RIYLVFEYLDLDLKKFMDSCPEFAKN   100
Ms        EISLLKEMQHRNI    VRLQDVVHSDK RLYLVFEYLDLDLKKHMDSSPEFIKD    97
Ara       EISLLKEMQHSNI    VKLQDVVHSEK RLYLVFEYLDLDLKKHMDSTPDFSKD   100

Sp        EISLLKEVNDENNRSNCVRLLDILHAES KLYLVFEFLDMDLKKYMDRISETGAT   104
Sc        EISLLKELKDDNI    VRLYDIVHSDAHKLYLVFEFLDLDLKRYMEGIPKDQPL   108
Hs        EISLLKELRHPNI    VSLQDVLMQDS RLYLIFEFLSMDLKKYLDSIPPGQYM   100

                                ********
                        ********
EG1       LALVKSYLFQLLQGLAFCHSHRVLHRDLKPQNLLINSDGA IKLADFGLAR      150
cdk2      LPLIKSYLFQLLQGLAFCHSHRVLHRDLKPQNLLINTEGA IKLADFGLAR      150
GF        LPLVKSYLFQLLQGLAFCHSHRVLHRDLKPQNLLINAQGE IKLADFGLAR
150

TaA       PALIKSYLYQILRGVAYCHSHRVLHRDLKPQNLLIDRRTNALKLADFGLAR      151
TaB       HHIVKSFLYQILHGIAYCHSHRVLHRDLKPQNLLIDRRTNSLKLADFGLAR      150
Os        PTLIKSYLYQILRGVAYCHSHRVLHRDLKPQNLLIDRRTNALKLADFGLAR      151
Zm        PTLIKSYLYQILHGVAYCHSHRVLHRDLKPQNLLIDRRTNALKLADFGLAR      151
Ms        PRQVKMFLYQMLCGIAYCHSHRVLHRDLKPQNLLIDRRTNSLKLADFGLAR      148
Ara       LHMIKTYLYQILRGIAYCHSHRVLHRDLKPQNLLIDRRTNSLKLADFGLAR      151

Sp   SLDPRLVQKFTYQLVNGVNFCHSRRIIHRDLKPQNLLIDKEGN LKLADFGLAR      157
Sc     GADIVKKFMMQLCKGIAYCHSHRILHRDLKPQNLLINKDGN LKLGDFGLAR      159
Hs     DSSLVKSYLYQILQGIVFCHSRRVLHRDLKPQNLLIDDKGT IKLADFGLAR      151


                          ↓     *********
EG1       AFGVPVRTFTHEVVTLWYRAPEILLGCKFYSTAVDIWSLGCIFAEMITRR      200
cdk2      AFGVPVRTYTHEVVTLWYRAPEILLGSKYYSTAVDIWSLGCIFAEMVTRR      200
GF        AFGVPVRTYTHEVVTLWYRAPEILLGCKYYSTAVDIWSLGCIFAEMITRK      200

TaA       AFGIPVRTFTHEVVTLWYRAPEILLGARQYSTPVDVWSVGCIFAEMVNQK      201
TaB       AFGIPVRTFTHEVVTLWYRAPEILLGARQYSTPVDVWSVGCIFAEMVNQK      200
Os        AFGIPVRTFTHEVVTLWYRAPEILLGSRQYSTPVDMWSVGCIFAEMVNQK      201
Zm        AFGIPVRTFTHEVVTLWYRAPEILLGARQYSTPVDVWSVGCIFAEMVNQK      201
Ms        AFGIPVRTFTHEVVTLWYRAPEILLGSRHYSTPVDVWSVGCIFAEMANRR      198
Ara       AFGIPVRTFTHEVVTLWYRAPEILLGSHHYSTPVDIWSVGCIFAEMISQK      201

Sp        SFGVPLRNYTHEIVTLWYRAPEVLLGSRHYSTGVDIWSVGCIFAEMIRRS      207
Sc        AFGVPLRAYTHEIVTLWYRAPEVLLGGKQYSTGVDTWSIGCIFAEMCNRK      209
Hs        AFGIPIRVYTHEVVTLWYRSPEVLLGSARYSTPVDIWSIGTIFAELATKK      201
```

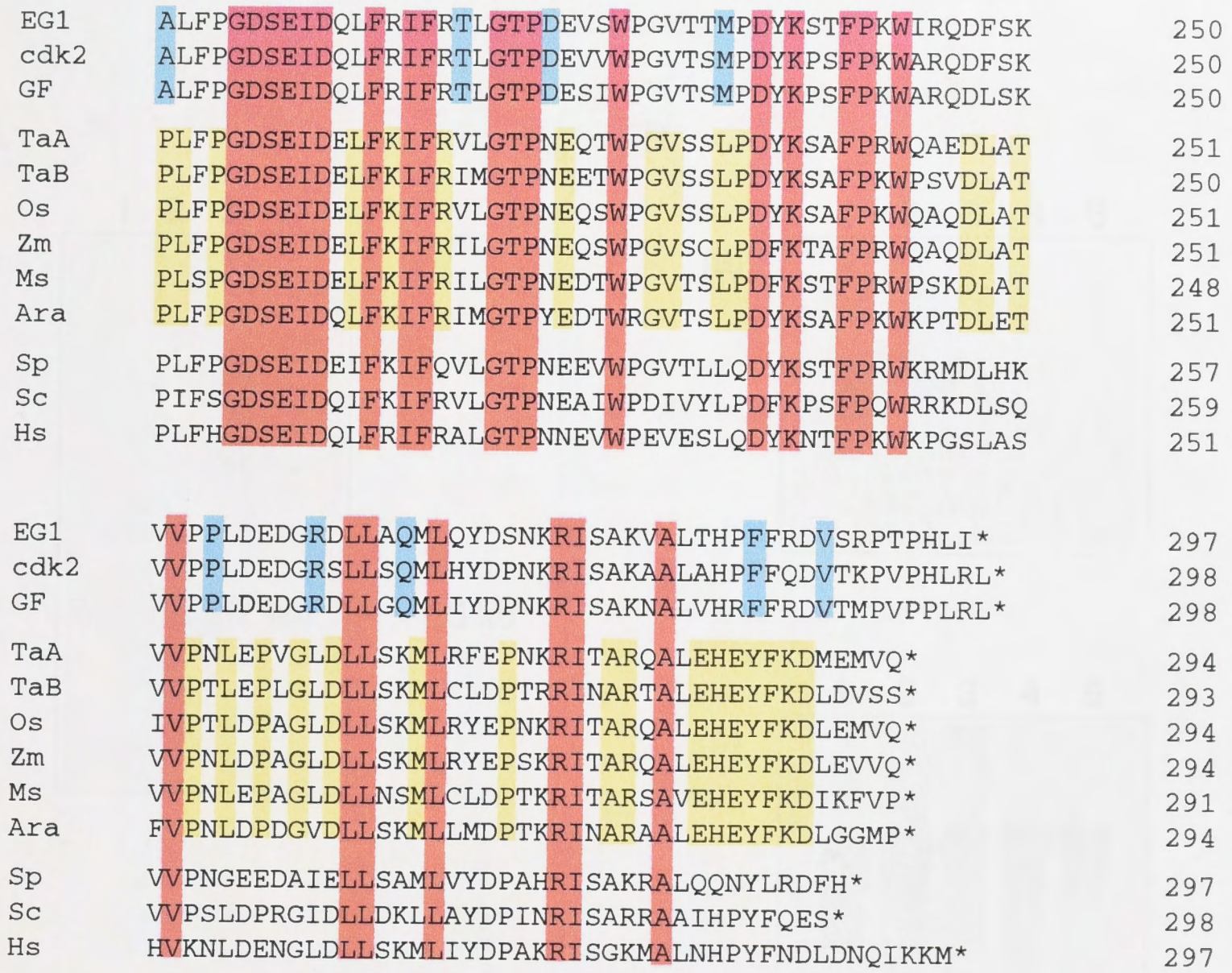


Fig. 3.12 Sequence alignment of the *cdk2* genes from human, *Xenopus* and Goldfish (top 3 sequences) with functional *cdc2* homologues from plants (middle 6 sequences), and with *cdc2* from human and yeasts (bottom 3 sequences).
The wheat *cdc2*-like genes, *cdc2TaA* and *cdc2TaB* (TaA and TaB), are compared with the *cdc2* homologues of rice (Os); maize (Zm); alfalfa (Ms); *Arabidopsis* (Ara); *S. pombe* (Sp); *S. cerevisiae* (Sc) and human (Hs).Also shown are *cdk2* sequences of Human (sdk2); Xenopus (Eg1) and goldfish *cdk2* (GF), which are listed above the *cdc2* homologues. All sequences are derived from the GenBank database. Red boxes indicate amino acids that are conserved in all *cdc2* and *cdk2* sequences. Yellow boxes indicate amino acids that are conserved in all plant *cdc2*-like genes. Blue boxes indicate amino acids conserved in all *cdk2* genes but are different in *cdc2* homologues. Asterisks indicate the regions selected for the design of the *cdc2* PCR primers.The arrows indicate Thr14, Tyr15 and Thr161 of *cdc2* (which is equal to Thr167 in *S.pombe*) which amino acids are important sites of phosphorylation in the control of $p34^{cdc2}$ protein kinase enzyme activity.

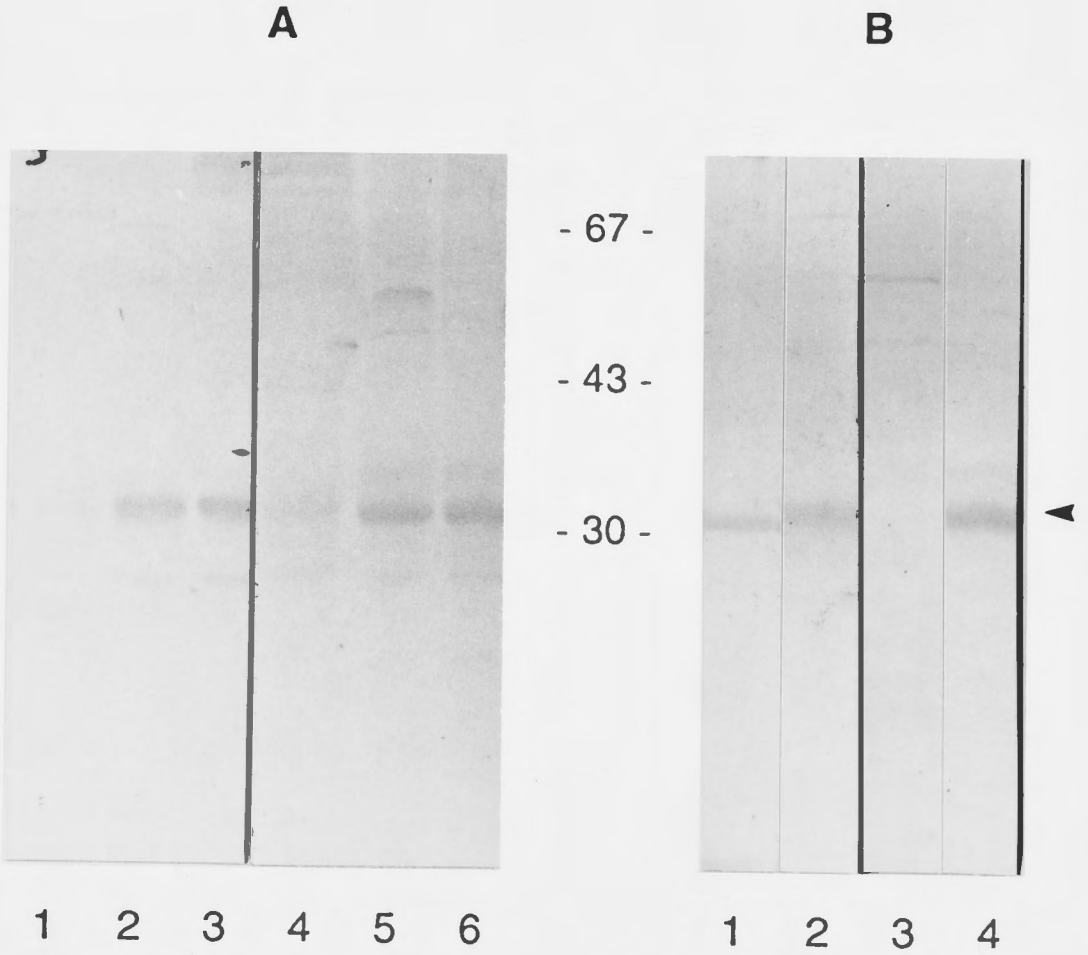


Fig. 3.14 Detection of p34^{cdc2}-like protein in wheat meristem by Western blotting.

A, extracts from 7-day-old wheat leaf tip (lanes 1 and 4), leaf meristem (lanes 2 and 5) and wheat root tip meristem (lanes 3 and 6), were Western blotted and probed with anti-PSTAIR antibody, lanes 1, 2, 3; or with anti-cdc2TaB antibody (an antiserum against the carboxy-terminal peptide of Cdc2TaB), lanes 4, 5, 6. The same band of about 34 kDa was detected by both antibodies in wheat leaf and root meristem.
B, extracts from yeast *S. cerevisiae* (wild type strain w303, lanes 1 and 3) and from wheat leaf meristem (lanes 2 and 4), were Western blotted and probed with anti-PSTAIR antibody, lanes 1 and 2; or with anti-cdc2TaB antibody (see above), lanes 3 and 4. Anti-cdc2TaB antibody is seen to be specific for wheat Cdc2-like protein. Arrow indicates wheat Cdc2-like protein, which ran a little more slowly then yeast Cdc28 protein.

Table 3.1 Degenerate oligonucleotide primers designed from highly conserved regions of *cdc2* genes (see Fig. 3.12) for PCR amplification of wheat *cdc2*-like genes. Uppercase lettering indicates nucleotide sequence corresponding to a highly conserved amino acid sequence and lowercase indicates the restriction site sequence incorporated at the 5' end of primers to facilitate cloning. An *EcoRI* site flanks the forward primers (5' primers) and a *BamHI* site flanks the reverse primers (3' primers).

Name	Oligonucleotide sequence	Amino acid sequence
5'cdc2/I	ggg gaa ttc GAA GGA ACA TAC GGA GTA GTA TA G T T T T T T C C C C C G G G G G	E G T Y G V V Y
5'cdc2/II*	ccg gaa ttc GAG GGC GTC CCG TCC ACC GCA ATC C C	E G V P S T A I
3'cdc2/I	ggg gga tcc ATT TTG TGG CTT TAA GTC TCT GTG G C A T C G A C G A C A A G G G	H R D L K P Q N
3'cdc2/II*	cgc gga tcc AGA AGG ATC TCT GGA GCT CTG TAC CA C A T A	W Y R A P E I L L

* 5'cdc2/II and 3'cdc2/II are almost unique primers based on the nucleotide sequence of two monocotyledonous cdc2 genes; rice *cdc2-1* and maize *cdc2A*.

Table 3.2 Amino acid sequence identity (%) between wheat p34*cdc2* proteins and other p34*cdc2* kinase homologues.

	cdc2TaA	*cdc2TaB*	*Spcdc2*	*ScCDC28*	*Hscdc2*	*Dmcdc2*	*Ggcdc2*	*Mmcdc2*	*XlEgI*	*Hscdk2*
cdc2TaA	100	85.1	61.9	59.9	63.4	60.9	61.8	63.2	66.6	66.0
cdc2TaB	85.1	100	60.1	61.1	63.1	59.5	62.2	63.2	63.9	63.7

	Aracdc2a	*Mscdc2A*	*Mscdc2B*	*Zmcdc2A*	*Oscdc21*	*Oscdc22*	*Gmcdc2S5*	*Gmcdc2S6*
cdc2TaA	82.4	82.4	85.1	92.5	92.5	84.1	84.1	85.1
cdc2TaB	84.4	84.1	85.1	85.0	83.7	91.8	85.8	85.8

The extent of sequence identity (%) was obtained by pairwise alignment, in GCG software, of the proteins encoded by wheat *cdc2*-like genes (*cdc2TaA*, *cdc2TaB*, using the full length Open-reading-frame of *cdc2TaB* by incorporating the RACE-B carboxyl-terminal sequence), compared with *S. pombe cdc2* gene (*Spcdc2*, Hindley and Phear,1984); *S. cerevisiae CDC28* gene (*ScCDC28*, Lorincz and Reed, 1984); human *cdc2* gene (*Hscdc2*, Lee and Nurse, 1987); *Drosophila cdc2* gene (*Dmcdc2*, Lehner and O'Farrell, 1990); chicken *cdc2* gene (*Ggcdc2*, Krek and Nigg, 1989); mouse *cdc2* gene (*Mmcdc2*, Th'ng et al., 1990); *Xenopus cdk2* gene (*XlEg1*, Paris et al., 1991); human *cdk2* gene (*Hscdk2*, Elledge and Spottswood, 1991); *Arabidopsis cdc2a* gene (*Aracdc2a*, Ferreira et al, 1991); alfalfa *cdc2* genes (*Mscdc2A*, *Mscdc2B*, Hirt et al., 1991,1993); maize *cdc2A* gene (*Zmcdc2A*, Colasanti et al., 1991); rice *cdc2* genes (*Oscdc21* and *Oscdc22*, Hashimoto et al., 1992); and soybean *cdc2* genes (*Gmcdc2S5* and *Gmcdc2S6*, Miao et al., 1993).

Chapter 4
Test of Functional Homology by Complementation of *cdc28^{ts}* Mutants

4.1 Introduction

The first direct evidence for the universality of cell cycle control proteins was the complementation of a *cdc2^{ts}* mutant in *S. pombe* with the *CDC28* gene of *S. cerevisiae* (Beach et al., 1982). Since then complementation has been used to isolate *cdc2* and other genes and also to test the functional homology of putative equivalent genes. The human *cdc2* gene was isolated by complementation of *cdc2^{ts}* mutant (Lee and Nurse, 1987). Chicken and *Drosophila cdc2*-like genes have proven to be functional homologues by complementation *cdc2^{ts}/cdc28^{ts}* mutants (Krek and Nigg, 1989; Lehner and O'Farrell, 1990). Plant *cdc2* homologues can also rescue *cdc2^{ts}* mutants or *cdc28^{ts}* mutants, for example, *cdc2*-like genes from *Arabidopsis*, alfalfa, rice, maize and soybean have complemented *cdc28^{ts}* mutants (Ferreira et al., 1991; Hirt et al., 1993; Hashimoto et al., 1992; Colasanti et al., 1991; Miao et al., 1993), and *Arabidopsis cdc2a* and alfalfa *cdc2MsA* gene also can complement *cdc2^{ts}* mutants (Hirayama et al., 1991; Hirt et al., 1993). These instances indicate that the *cdc2* gene has been functionally conserved during evolution of the eukaryotes.

In yeasts, a single p34*$^{cdc2/CDC28}$* protein kinase plays a master role in the cell cycle, by assembling sequentially into active holoenzyme complexes with different cyclins that are abundant in G1, S, or mitotic phases and driving key events at the G1-S phase transition and then separate later events at mitosis. For example in budding yeast, Cln1, Cln2 and Cln3 are G1 cyclins (Hadwiger et al., 1989b; Richardson et al., 1989), Clb5 and Clb6 are S phase cyclins (Epstein and Cross, 1992; Schwob and Nasmyth, 1993) and in association with Cdc28 are believed to promote events in late G1, S phase and G2 phase,

while Clb1 and Clb2 are mitotic cyclins (Surana et al., 1991). In fission yeast the G1 cyclins have not been fully characterised but p56^{cdc13} is a mitotic cyclin of B-type which associates with p34^{cdc2} during late G2 and mitosis (Moreno et al., 1989). p13^{suc1} is also found to be physically associated with p34^{cdc2} and is essential for completion of mitosis (Brizuela et al., 1987; Booher et al., 1989). A further aspect of the control of p34^{cdc2} kinase is its regulation by enzymes that influence the phosphorylation status of threonine and tyrosine. Enzymes that control this phosphorylation have been described in Chapter1 (1.5).

The precise function of *cdc2*-like genes of plants is of interest because in vertebrates, instead of a single p34^{cdc2} protein involved in the core events of DNA replication and segregation, there are multiple *cdc2*-like proteins, for example, *cdk2*, *cdk3*, *cdk4*, *cdk5* and *cdk6* are all found in human cells in addition to *cdc2* (Meyerson et al., 1992; Matsushime et al., 1992; reviewed by Pines 1995). There is also a greater diversity of G1 cyclins in vertebrates, in proportion with the number of CDKs, and both perhaps associated with the G0 to G1 phase transition that does not occur in yeasts. The G0 to G1 phase transition is tightly regulated by growth hormones in multicellular animals so preventing, in non-malignant cells, the unrestrained proliferation that could otherwise result from nutrient-rich body fluids. The mammalian G1 cyclins, C, D and E, are likely candidates for function in the G0 to G1 and G1 to S transitions and were isolated through their capacity to rescue a triple *CLN*-defective yeast strain. Three D-type cyclins (D1, D2 and D3) act at early G1 by associating with either Cdk4 or Cdk6, and cyclin E combines later in G1 with Cdk2 (reviewed by Sherr 1993, 1994). Once cells enter S phase, cyclin E is degraded and Cdk2 next complexes with cyclin A, so cyclin A may have the function of sustaining DNA synthesis. Subsequently initiation of mitosis by Cdc2 in association with cyclin B parallels the yeast cell cycle and can be considered a universal mechanism (Nurse 1990). Cdk2 of animals is however much closer in function to p34^{cdc2} of yeast cells than the CDKs that act early in G1 phase and can be thought of as having taken over the G1-S phase functions of p34^{cdc2}.

I have cloned two wheat *cdc2*-like genes (see Chapter 3) which showed strong structural similarity with other *cdc2* homologues, especially with plant *cdc2* homologues.

To further test whether these *cdc2*-like genes do encode proteins capable of carrying out the essential functions of p34$^{cdc2/CDC28}$ or perhaps the related more restricted functions of Cdk2, I tested their capacity for complementation of *cdc28*^{ts} mutants.

Mutant alleles of fission yeast *cdc2*^{ts} usually cause arrest at both G1-S and G2-M transitions (Nurse and Bissett, 1981) although they were first recognised as predominantly causing arrest at G2-M because of the prolonged G2 phase in fission yeast and therefore the preponderance of cells remaining in G2 phase when asynchronous populations were shifted to restrictive conditions. Conversely the prolonged G1 phase of budding yeast caused asynchronous *cdc28*^{ts} cells to arrest mostly in G1 on transfer to restrictive temperature leading to a reluctance to accept a role for *CDC28* at mitosis (Hartwell et al., 1974; Reed 1980). One *cdc28*^{ts} mutant, *cdc28-1N*^{ts}, uniquely arrests at G2 phase at the restrictive temperature (Piggott et al., 1982), thereby providing an early indication of the equivalence of *cdc2* and *CDC28* genes and of the universality of eukaryote cell cycle catalysts which was dramatically confirmed by the complementation of *cdc2*^{ts} by *CDC28* (Beach et al., 1982).

A *cdc2*^{ts} mutant causing arrest only at G1-S is not available. I chose budding yeast *cdc28*^{ts} mutants for complementation studies because they allowed testing for complementation of G1-S and G2-M functions and also because completion of the G1-S transition is signalled by the appearance of a bud almost at the same time as initiation of DNA synthesis (Hartwell et al., 1974). Therefore using budding yeast the function of a foreign gene at G1-S or G2-M could be judged.

4.2 Results

4.2.1 Introduction of *cdc2TaA* and *cdc2TaB* cDNA into a vector for expression in yeast cells

To test whether wheat *cdc2*-like genes can perform the functions of the yeast *CDC28/cdc2* gene, I tested for complementation of temperature-sensitive *S. cerevisiae* *cdc28* mutants, *cdc28-4*^{ts}, *cdc28-13*^{ts}, and *cdc28-1N*^{ts}. These cause arrest at both G1-S

and G2-M in the case of *cdc28-4*^{ts} and *cdc28-13*^{ts} (Reed 1980; Reed and Wittenberg, 1990), or at G2-M only with *cdc28-1N*^{ts} (Piggott et al., 1982). These mutations have been used successfully for complementation tests of *cdc2* homologues of alfalfa, maize, rice and soybean (documented in 4.1). For this purpose, I inserted cloned wheat *cdc2*-like cDNAs into the *S. cerevisiae* expression vector pYES2 to allow expression under control of the inducible *GAL1* promoter.

The pYES2 vector (Invitrogen) is a high copy yeast/*E. coli* shuttle vector that has been constructed with upstream promoter sequences from the *GAL1* gene and transcription termination signals from the *CYC1* gene of *S. cerevisiae*. It also contains the *S. cerevisiae URA3* gene to provide a selectable marker for use in host strains of *ura3*$^{-}$ genotype (Fig. 2.3).

For the purpose of expression in yeast, the putative p34^{cdc2TaA} coding cDNA was inserted downstream of the *GAL1* promoter of the pYES2. The small upstream ORF (Chapter 3) was discarded for this test. It was found that a *BsrB*I restriction site was located at cDNA nucleotide position 80 upstream of the presumed p34^{cdc2TaA} initiation codon ATG. Therefore, the cDNA of *cdc2TaA* was isolated from pBluescript/cdc2TaA plasmid by digestion with restriction enzyme *BsrB*I, together with *Xho*I to cut a site in pBluescript downstream of the 3' end of the cDNA. The excised *BsrB*I-*Xho*I fragment had a blunt and sticky end. This fragment was subcloned into pBluescript cut at *Sma*I and *Xho*I sites. The insert DNA was then cut with *BamH*I and *Xho*I, and cloned into the pYES2 plasmid at *BamH*I and *Xho*I sites. The recombinant plasmid, pYES2/cdc2TaA, had an insert of *cdc2TaA* cDNA in sense orientation downstream from the *GAL1* promoter and upstream of the termination signal of *CYC1* (see Fig. 4.1).

Since *cdc2TaB* was incomplete in the cDNA library, lacking 13 amino acids at its C-terminus, while the RACE-B PCR product of *cdc2TaB* was complete at its C-terminus but was incomplete at its N-terminus, the strategy for expressing a full-length coding cDNA of *cdc2TaB* was to combine *cdc2TaB* with RACE-B. The extensive overlap of 534 bp confirmed that these two elements were from a single gene and could legitimately be combined to form a full length coding cDNA. The validity of this strategy, based on the extensive overlap region and the small amount sequence based only on RACE-B, has

been discussed earlier in Chapter 3 (3.2.3). For the splicing a *Hind*III site in the overlapping region of *cdc2TaB* and RACE-B was chosen for connecting the two pieces of cDNA. The *cdc2TaB* was cut with *EcoR*V and *Hind*III, while RACE-B was cut with *EcoR*I and *Hind*III, then these fragments and linearised pBluescript SK$^+$ cut with *Sma*I-*EcoR*I were ligated together to form a full-length *cdc2TaB* cDNA subcloned in pBluescript SK$^+$. This full-length *cdc2TaB* was then subcloned into pYES2 at *EcoR*I site. The recombinant plasmid, pYES2/cdc2TaB, with sense orientation of *cdc2TaB* relative to the *GAL1* promoter, was selected from among recombinant clones by restriction mapping with *BamH*1 and *Hind*III, using the formation of a 500 bp band to indicate a recombinant plasmid with the right orientation (see Fig. 4.2).

4.2.2 *cdc2TaA* can partially complement *cdc28-13^{ts}* and *cdc28-1N^{ts}*, but *cdc2TaB* cannot

S. cerevisiae CDC28 mutant strains, *cdc28-4^{ts}*, *cdc28-13^{ts}* and *cdc28-1N^{ts}*, carry temperature-sensitive mutations in the *CDC28* gene that cause different phenotypes. All can grow at a permissive temperature of 28 °C and are blocked in division at the restrictive temperature of 37 °C, however *cdc28-4^{ts}* and *cdc28-13^{ts}* block predominantly in G1 phase on transfer of an asynchronous culture to restrictive temperature (Reed 1980), but *cdc28-1N^{ts}* blocks in G2 phase being able to carry out the G1-S transition but not mitosis (Piggott, 1982). The phenotypes of mutants *cdc28-4^{ts}* and *cdc28-13^{ts}* on detailed analysis have been found to include loss of ability to pass through mitosis at the restrictive temperature (Reed and Wittenberg, 1990). The predominance of arrest in G1 phase from asynchronous culture simply reflects the fact that G1 is the longest phase in this yeast and cells in G1 are more numerous in an asynchronous culture at time of temperature shift. All of the mutants used to test complementation carried a *ura3$^-$* mutation which allowed the selection of pYES2 transformants.

These *cdc28^{ts}* mutants were transformed with pYES2/cdc2TaA, pYES2/cdc2TaB and as a control with unmodified pYES2. Transformants were first selected for uracil prototrophy at the permissive temperature of 28 °C for 3 days. For each wheat gene

four clones were selected to test for complementation of the *cdc28^{ts}* mutation by induction of the wheat gene at a temperature restrictive for *cdc28^{ts}* function. For tests on solid media, transformants were plated on to galactose agar to induce expression from the *GAL1* promoter, incubated first at 28 °C for 3-5 h, then transferred to 37 °C for 4 days. As a control, transformants were also plated onto glucose agar to repress the *GAL1* promoter, and were incubated at 28 °C and 37 °C using the same condition as for galactose plates. After 4 days incubation, *cdc28-13^{ts}* and *cdc28-1N^{ts}* cells that had been transformed with pYES2/cdc2TaA were able to form colonies at 37 °C on galactose medium (Fig. 4.3). Whereas the same mutants carrying only pYES2 without plant genes were not able to form colonies. All transformants were able to grow at 28 °C at which temperature the mutant Cdc28 enzymes were active and colonies appeared after 2 days incubation at this temperature. This colony formation occurred earlier than with mutants *cdc28-13^{ts}* and *cdc28-1N^{ts}* complemented by *cdc2TaA* at 37 °C, which took 4 days, therefore indicating that complementation was imperfect. On glucose medium all transformants could grow at 28 °C, but not at 37 °C (Fig. 4.3), confirming that back mutation of the *CDC28* genes had not occurred. It can be concluded that wheat *cdc2TaA* but not *cdc2TaB* is able to substitute for the mutated *cdc28* protein in *cdc28-13^{ts}* and *cdc28-1N^{ts}* strains.

To further confirm that cell proliferation by transformants of *cdc28-13^{ts}* and *cdc28-1N^{ts}* at 37 °C was really plasmid-dependant, the effect of plasmid loss was tested. To allow loss of plasmid the transformants were grown for 24 h at 28 °C in rich liquid medium (YEPD) without selection for uracil prototrophy. The cells were then diluted and plated on to YEPD plates to give about 100 colonies per plate. After incubation at 28 °C for two days, colony replicas were plated onto minimal media with or without uracil, and incubated at 28 °C for one day. Due to plasmid loss, only about 50% of colonies could grow on plates without uracil although all grew with uracil. Ten individual colonies with plasmid and ten without plasmid were randomly selected to test for complementation of Cdc28 defects at 37 °C and were streaked onto galactose medium supplemented with uracil. After incubation at 37 °C for 2 days, the cells with plasmid had all been able to form colonies, whereas cells without plasmid did not

proliferate (Fig. 4.4). It can therefore be concluded that the ability of *cdc28-13^{ts}* and *cdc28-1N^{ts}* mutants to grow at 37 °C was conferred by the wheat *cdc2TaA* gene carried on the pYES2 plasmid.

Immunoblots confirmed that the *cdc2TaA* and *cdc2TaB* protein was expressed after induction with galactose, but the amount of *cdc2TaA* protein accumulated a little more than that of *cdc2TaB* protein judging by Western blots carrying equal amounts of total yeast protein (Fig. 4.9). The rabbit antiserum used in these blots was raised against a 14 amino acid-C-terminal peptide of *cdc2TaB* and was unambiguously revealed, by this test of extracts containing products of each wheat *cdc2* gene separately, to be able to detect both wheat *cdc2*-like proteins. This confirmed the successful expression of both of the wheat *cdc2*-like genes under conditions of complementation testing.

4.2.3 Analysis of complementation by use of liquid culture and flow cytometry

Growth of colonies on plates provided an indication that complementation was incomplete. To test this further, liquid culture was used to test rates of division and prevalence of cell cycle phases.

cdc28-13^{ts} and *cdc28-1N^{ts}* carrying pYES2/cdc2TaA, or controls with only pYES2, together with a wild type strain w303 were grown in minimal liquid medium at 28 °C. After 2 days the cells of conditional mutants were in exponential phase. This period was longer than that required by the wild type cells, which grow overnight, and indicated that the altered Cdc28 protein is not fully functional even at permissive temperature. Cells in exponential phase were washed and resuspended in a galactose medium to a density of 4 x 10^6/ml and grown at 37 °C (see method in Chapter 2, 2.2.14.4). In the first 12 h at restrictive temperature, the difference between cells with pYES2/cdc2TaA and with unmodified pYES2 was not obvious (Fig. 4.5). After 12 h incubation at 37 °C, the cell number in culture of the mutants carrying pYES2/cdc2TaA clearly began to increase while controls with unmodified pYES2 did not (see Fig. 4.5, 4.6, 4.7). The rate of division in cells complemented by wheat genes was slow. More effective

complementation of yeast mutants has been reported for *cdc2* homologues of *Drosophila,* alfalfa, soybean (Lehner and O'Farrell, 1990; Hirt et al., 1993; Miao et al., 1993), with which complementation became apparent between 3-10 h when cultured at restrictive temperature. However slow or partial complementation by plant *cdc2* genes has also been noted (Ferreira et al., 1991; Hirayama et al., 1991). The slow progress of complemented cell proliferation observed in liquid culture was consistent with the observation that mutants carrying pYES2/cdc2TaA, when plated on agar at restrictive temperature, required 4 days for colony formation compared with only 2 days at permissive temperature.

Fig. 4.5 also shows that mutant *cdc28-13^{ts}* was more effectively complemented by *cdc2TaA* than was *cdc28-1N^{ts}*. This is consistent with the earlier observation (Fig. 4.3) that *cdc28-13^{ts}* harbouring pYES2/cdc2TaA could form larger colonies on agar than could *cdc28-1N^{ts}* containing the same plasmid. These two mutants of *cdc28^{ts}* may differ in the severity with which *CDC28* function is affected, as well as in the identity of the function that has been disabled by mutation. When preparing yeast cells for transformation, I observed that *cdc28-13^{ts}* grew more rapidly in liquid culture than did *cdc28-1N^{ts}* and *cdc28-4^{ts}* in the same conditions. In particular *cdc28-4^{ts}* needed twice as long to reach the desired turbidity. In summary *cdc28-13^{ts}* grew fastest, while *cdc28-4^{ts}* grew slowest among the three mutants. This observation must be interpreted with caution in terms of complementation because there are at least two factors that may be relevant; one is the importance of the functions that may have been partially or wholly lost, the other is the extend to which the Cdc28 protein loses activity between permissive and restrictive temperatures. Loss of activity may be more or less complete regardless of the nature of the function(s) of Cdc28 that have been affected. The greater capacity of Cdc2TaA to complement *cdc28-13^{ts}* than *cdc28-1N^{ts}* (Fig. 4.3, 4.5) could be taken to indicate that the wheat gene can supply the G1 functions lost by *cdc28-13^{ts}* better than the G2 functions lost by *cdc28-1N^{ts}*, however this need not indicate that Cdc2TaA has specifically G1 functions since it is unable to complement *cdc28-4^{ts}*, which also arrests in G1 phase. A simple explanation may be that *cdc28-4^{ts}* more completely loses its activity at 37 °C, or that the inactivated protein has dominant negative

interactions with potentially active Cdc28 proteins. Thus the lesion of *cdc28* in *cdc28-4^{ts}* may be more severe than that in *cdc28-13^{ts}* and *cdc28-1N^{ts}*, with the result that the *cdc2TaA* could partially rescue *cdc28-13^{ts}* and *cdc28-1N^{ts}*, but not *cdc28-4^{ts}*. This partial rescue of both G1-arresting and G2-arresting mutants is more simply explained if TaA is a *cdc2*-like gene rather than of a more restricted function like Cdk2 (see discussion).

Morphological observation of complemented *cdc28^{ts}* mutants grown at restrictive temperature revealed a lag period before complementation could be detected and confirmed that the cell cycle did not occur with normal kinetics in *cdc28-13^{ts}* and *cdc28-1N^{ts}* rescued with Cdc2TaA. *cdc28-13^{ts}* cells carrying pYES2/cdc2TaA remained mostly unbudded during the first 12 h of incubation at 37 °C in galactose medium (Fig 4.6, a and b). The cells had therefore not progressed from G1 phase (Hartwell et al., 1974; Reed 1980). It is probable that accumulation of sufficient Cdc2TaA to rescue the *cdc28-13^{ts}* cells needed a significant period of time since incubation for 24 h in galactose medium at 37 °C was necessary before some cells formed elongated buds indicating that START had been traversed and by 48 h (Fig 4.6 c) most cells had large buds indicating that progress through mitosis and daughter cell formation was slower than in wild-type cells (Fig. 4.7 c and d). Many cells (Fig. 4.6 c) had aberrant elongated buds and some daughter cells apparently failed to separate completely yet budding continued, thus, forming multiple buds, which is consistent with capacity to execute START. In contrast, the *cdc28-13^{ts}* cells carrying unmodified pYES2 remained round and unbudded and their number did not increase (Fig. 4.6 d and e). This shows clearly that in the absence of Cdc2TaA expression, the *cdc28-13^{ts}* cells blocked in G1 phase and could not execute START and bud emergence.

Although in *cdc28-13^{ts}* Cdc2TaA appeared to preferentially complement G1 to S progression (signalled by bud emergence after passing START) the plant gene was also able to complement loss of function in *cdc28-1N^{ts}*, which causes predominantly a loss of capacity for mitosis. After incubation in galactose medium for 12 h, *cdc28-1N^{ts}* cells carrying pYES2/cdc2TaA had elongated buds and some of them had unseparated daughter cells (similar to complemented *cdc28-13^{ts}*, Fig. 4.7 a, b) however there was an

increase in cell number indicating that some divisions were completed within 12 h (Fig. 4.5), and this was due to Cdc2TaA since *cdc28-1N^{ts}* cells carrying unmodified vector did not increase in cell number and had elongated buds characteristic of the mitotic defect in this allele.

Flow cytometric analysis of propidium iodide-stained *cdc28-13^{ts}* cells (Fig. 4.8) also showed that, although cells could proliferate at 28 °C, an unusually large proportion were in G1 phase at that temperature (Fig. 4.8 a and d, 0 h) indicating a poor capacity for G1 phase to S phase progression at permissive temperature. After incubation in galactose medium to induce the plant enzyme, cells carrying pYES2/cdc2TaA showed a restored small peak of G2 phase cells (Fig. 4.8 c), whereas cells carrying unmodified pYES2 remained in G1 phase (Fig. 4.8 e, f). In the later samples of cells lacking a complementing gene (Fig. 4.8, f) there is evidence of cell death giving rise to small fragments and the rightward drift of the main peak was due to cell enlargement and cytoplasmic autofluorescence. The appearence of a detectable peak of cells in G2 phase indicates that there was in complemented cells (Fig. 4.8 c) an increase in the rate with which cells could traverse G1-S relative to the rate of G2-M progress, compared with rates in cells growing at the permissive temperature (Fig. 4.8 a, d). This is most simply explained as the wild type wheat protein being more effective in G1-S processes than the mutant Cdc28 protein of the *cdc28-13^{ts}* allele.

4.3 Discussion

To investigate the functional homology of wheat *cdc2*-like genes, complementa-tion of budding yeast *cdc28^{ts}* mutants was assessed in this Chapter. Expression of wheat *cdc2TaA* cDNA in *S. cerevisiae* mutants *cdc28-13^{ts}* and *cdc28-1N^{ts}* promoted cell division at the restrictive temperature, indicating that the protein encoded by the *cdc2TaA* is a functional homologue of p34^{cdc2}.

The *cdc28*^{ts} mutants used in this study were *cdc28-4*^{ts}, *cdc28-13*^{ts} and *cdc28-1N*^{ts}. The phenotype of *cdc28-4*^{ts} and *cdc28-13*^{ts} is that division in cells transferred from asynchronous culture becomes blocked predominantly in G1 phase at the restrictive temperature (Reed, 1980), however analysis by using inhibitors to cause arrest in S or G2 phase at permissive temperature and then shifting to a high restrictive temperature (38 °C) and releasing the S or G2 phase inhibitor, has showed these mutants can homogeneously arrest in G2 phase, indicating they are also defective in G2-M function (Reed and Wittenberg, 1990). The phenotype of *cdc28-1N*^{ts} is that the cell cycle is blocked in G2 phase at the restrictive temperature (Piggott et al., 1982). Taking these phenotypes together it is clear that *CDC28* is required at both G1-S and G2-M transitions. The *cdc2TaA* gene was found here to promote *cdc28-13*^{ts} and *cdc28-1N*^{ts} proliferation at the restrictive temperature (Figs. 4.3, 4.4, 4.5), although the complementation did not fully restore the wild type rate of progress through division events, resulting in cells becoming large and bearing large and sometimes multiple buds (Figs. 4.6, 4.7). However it can be concluded from the restoration of division that *cdc2TaA* together with a possible residual contribution from Cdc28^{ts} can catalyse the essential functions of *CDC28* in both G1 and G2 phase.

The phenomenon of incomplete complementation has also been observed in the complementation studies of *Drosophila cdc2* homologues (Lehner and O'Farrell, 1990), *Arabidopsis cdc2* homologue (Ferreira et al., 1991; Hirayama et al., 1991) and rice *cdc2* homologues (Hashimoto et al., 1992). In *Arabidopsis*, Cdc2Ara could rescue a *cdc28*^{ts} mutant, but it did not restore the wild type rate of division, and prolonged incubation in galactose caused reduced viability and an increasing number of cells with aberrant morphology of the complemented yeast cells (Ferreira et al., 1991). Rice *cdc2Os-1* could partially complement *cdc28-4*^{ts} mutants but did not restore the complete wild type phenotype (Hashimoto et al., 1992). Partial complementation may suggest that wheat p34^{cdc2} protein does not interact normally with other components of the cell cycle control apparatus such as cyclins, Suc1, Wee1, Cdc25 and other kinases and phosphatases, which are required for activation of p34^{cdc2} kinase and completion of the cell division (see Chapter 1, 1.5).

The present evidence for plant genes encoding *cdc2*, cyclins, MAP kinase and protein phosphatase 1 (see Chapter 1, 1.7), suggests that the mechanism of the plant cell cycle is similar to that of yeasts and animals. Plants have yielded a number of cDNA sequences encoding plant mitotic cyclins with A- or B-type characteristics or having mixed A- and B-type features from carrot (Hata et al., 1991), Soybean (Hata et al., 1991), *Arabidopsis* (Hemerly et al., 1992), alfalfa (Hirt et al., 1992), *Antirrhinum* (Fobert et al., 1994) and maize (Renaudin et al., 1994). Recently, D-type cyclins have been found in *Arabidopsis* (Soni et al., 1995). Unlike *cdc2* homologues, which show extensive sequence similarity, cyclins are found to be diverse in extensive regions, indicating that they may have species-specific features besides the common CDK-binding function.

Complementation is an objective test for functional homology. But if a *cdc2*-like gene complements $cdc2^{ts}/cdc28^{ts}$ mutants only incompletely, it cannot be concluded that the gene is not homologous since it may have full $p34^{cdc2}$ function in the organism from which it is obtained and function less well in the test organism because less able to associate perfectly with other components, like cyclins, which may be species-specific.

It was observed that $cdc28\text{-}4^{ts}$ grew most poorly among three $cdc28^{ts}$ mutants at the permissive temperature, which suggests that lower activity reside in this mutant protein even at permissive temperature. All three mutants grew slower than wild type even at permissive temperature and abnormal cell cycle progress was also observed in these mutants (Fig. 4.6 a; Fig. 4.7 a; Fig 4.8 a, d). In $cdc28\text{-}13^{ts}$ the extreme predominance of G1 phase cells indicated the slow progress through G1-S and possibly an accelerated mitosis (Fig. 4.6 a; Fig. 4.8 a, d). While in $cdc28\text{-}1N^{ts}$ the high frequencies of cells with enlarged buds indicated slow progress through G2-M (Fig. 4.7 a). Interestingly the expression of wheat *cdc2* to some extent corrected the over-predominance of G1 phase cells in $cdc28\text{-}13^{ts}$ (Fig. 4.8 c). Lorincz and Reed (1986) have suggested that aberrant cell morphology and slower growth at permissive temperature in mutants is due to constitutively low activity of $p34^{cdc2}$; differences in mutant cell morphology may relate to differences in residual *in vivo* activity remaining at both permissive and restrictive temperature. Assay of H1 histone kinase activity in extracted $p34^{cdc2}$ enzyme confirms

both the difference in inherent activity and the difference in thermolability of different allelic forms of *S. pombe* p34^{cdc2} (Moreno et al., 1989). Similarly in *S. cerevisiae in vitro* p34^{cdc28} kinase activity from *cdc28-13^{ts}* and *cdc28-4^{ts}* was labile; the protein kinase activity of *cdc28-13^{ts}* was at the similar level to wild type protein at permissive temperature and much reduced at restrictive temperature (Reed et al., 1985) however the imbalance of G1 and G2 phases suggests that activity to some natural substrates may be low at the permissive temperature. The activity of *cdc28-4^{ts}* has been reported as hardly detected even at the permissive temperature (Reed et al., 1985) and may explain the very slow growth of *cdc28-4^{ts}* at permissive temperature and the absence of complementation by *cdc2TaA* implying that complementation requires a contribution from residual activity of p34^{cdc28ts}. Similarly the poorer performance of *cdc28-1N^{ts}* at permissive temperature correlates with the small extent of its complementation by *cdc2TaA*. However the capacity of wheat *cdc2TaA* to complement *CDC28* alleles that lack G1 function (*cdc28-13^{ts}*) and also G2-M function (*cdc28-1N^{ts}*) argues that it can act at both cell cycle events and is more consistent with *cdc2* than cdk2 identity.

Interestingly, wheat *cdc2TaB* gene complemented neither the G1-arresting *cdc28-4^{ts}* and *cdc28-13^{ts}* nor the G2-arresting *cdc28-1N^{ts}*, even though it has a high degree of structural homology with *cdc2TaA* (85% identity in protein sequence) and with other *cdc2*-functional homologues (see chapter 3). In the search for an explanation of the difference in complementation of yeast mutations between the two wheat *cdc2*-like genes, I have examined the evidence concerning which amino acids are important for p34^{cdc2} function.

There have been several studies on mutations affecting the yeast *cdc2^{ts}/cdc28^{ts}* protein, which have identified amino acid essential for its function. This information throws light on the significance of the mutations that were used to test complementation and also on the possible significance of amino acid substitutions in the two wheat genes *cdc2TaA* and *cdc2TaB*. Fig. 4.10 shows there are 13 different substitutions of amino acids in *cdc2^{ts}* (MacNeill et al., 1991; Carr et al., 1989) and 6 different substitutions representing 11 mutants of *cdc28^{ts}* (Lorincz and Reed, 1986) that can cause temperature-sensitive mutant phenotypes. All of the nineteen mutated positions are

widely distributed over the length of the protein, and can be divided into three regions: (1) the ATP-binding site at N-terminus (conserved sub-domains I and II in protein kinases, Hanks et al., 1988), (2) the central core of the catalytic domain (sub-domains VI-IX), and (3) carboxy terminus region. These amino acid substitution have been interpreted as correlating with a predicted secondary structure (Lorincz and Reed, 1986; Carr et al., 1989) which is confirmed by the recent X-ray crystallography of Cdk2 (DeBondt et al., 1993; Jeffrey et al., 1995). Some of the mutations have been considered to lie in regions that may be involved in the interaction of the $p34^{cdc2}$ with proteins encoded by the *wee1*, *cdc13*(*or CLB*) and *suc1* genes (Carr et al., 1989; MacNeill et al., 1991). For example, *cdc2-33, cdc2-56, cdc2-L7* and *cdc2-M26/M55* (position 137-208) can be suppressed by overexpression of wild-type $p13^{suc1}$ (Hayles et al., 1986a), whereas other $cdc2^{ts}$ mutants could not be rescued by $p13^{suc1}$. This suggests that the region of 137-208 amino acids constitute a binding region for $p13^{suc1}$. This interpretation could be challenged however if mutation removed all $p13^{suc1}$ binding, in which case raised $p13^{suc1}$ levels could only suppress if $p13^{suc1}$ bound elsewhere on $p34^{cdc2}$. The positive temperature-sensitive mutants *cdc2-1w/2w* and *cdc2-3w* (position 146 and 67, respectively), which bring a premature advancement of mitosis, may imply these mutated positions are important in interaction with *wee1* kinase and *cdc13* cyclin gene products (Carr et al., 1989). This interpretation is consistent with insensitivity of *cdc2-1w* to changes in the level of expression of the negatively-acting *wee1* gene product (Russell and Nurse, 1987b) and the inability of *cdc2-3w* protein to interact with the mutant product of the *cdc13-117* allele, while over-expression of wild-type *cdc2* suppresses the partial-function mutant *cdc13-117* (Booher and Beach, 1987).

Some $cdc2^{ts}$ mutants are affected in the kinase catalytic domain (regions VI to IX, hanks et al., 1988), for example, *cdc2-33* (position 177) is located at highly conserved protein kinase sub-domain VIII, *cdc2-M26/M55* (position 133) and *cdc28-4* (position 122, numbering as in *S. pombe*) is located at sub-domain VI (Hanks et al., 1988); positions of these mutations are shown in Fig. 4.10. Some regions of Cdc2/Cdc28 are in common with other protein kinases. In particular, residues 9-38 of $p34^{cdc2}$ are homologous to the highly conserved ATP-binding site, and Lys33 is an invariant residue

among 65 different protein kinase (Hanks et al., 1988). Booher and Beach (1986) showed that an Arg instead of a Lys at position 33 can abolish p34^{cdc2} function.

Other regions are specific for the CDK class of protein kinases. Gould and Nurse (1989) have showed that a Tyr15 residue in the ATP binding domain is used in Cdc2 to control activity, being phosphorylated *in vivo* prior to mitosis and on dephosphorylation activating the *cdc2* kinase and triggering mitosis. Further studies have shown that Thr14 is also regulated by phosphorylation in animals reinforcing the Tyr15 phosphorylation control (Krek and Nigg, 1991). The phosphorylation of threonine 167 stimulates the mitotic activity of p34^{cdc2} (Gould et al., 1991; Ducommun et al., 1991; Lorca et al., 1992). Recent X-ray crystallography indicates that in Cdk2 the phosphorylation of Thr167 (= T161 in human p34^{cdc2} and T160 in p33^{cdk2}) would stabilise the T-loop (amino acids 145-170) through ionic interactions with a patch of basic residues in the carboxyl-terminal lobe of the protein, so making a partially activated cyclin-Cdk complex fully active (Jeffrey et al., 1995). Another CDK specific region is the 16 amino acid PSTAIR region, EGVPSTAIREISLLKE, (42-55) that is perfectly conserved in all p34^{cdc2} homologues and closely related enzymes such as Cdk2. This region is important for the interaction of p34^{cdc2} with cyclin subunits that are essential for its activity (Carr et al., 1989, Gautier et al., 1988; Jeffrey et al., 1995). These residues are shown in Fig. 4.10.

Considering the mutants used for complementation-testing in this study, mutant *cdc28-4^{ts}* has Tyr in place of wild-type His128. His128 is conserved in all Cdc2 homologues and is located at catalytic domain VI (Hanks et al., 1988), so mutation of *cdc28-4^{ts}* might be defective for protein kinase activity at the restrictive temperature. Mutant *cdc28-13^{ts}* has Gln in place of wild type Arg 283, which is invariant at the carboxy terminus of all *cdc2* homologues. Several other mutants are also found in the C-terminus region (see Fig. 4.10), so it indicates that the extreme carboxyl-terminus of the p34^{cdc2} protein is essential for its function. However antibodies directed against the extreme carboxyl-terminus of the fission yeast p34^{cdc2} protein do not inhibit p34^{cdc2} protein kinase activity measured *in vitro* (Simanis and Nurse 1986) which could indicate that this region is not essential but more likely indicates that when the carboxy terminal

region has favoured correct folding of the enzyme then that region of the surface can be obscured by antibody without detriment. Mutant *cdc28-1N*^{ts} has Leu in place of wild type Pro250, this residue of *S. pombe cdc2* if mutated by oligonucleotide-directed mutagenesis also abolished p34^{cdc2} function (Carr et al., 1989).

To assess possible differences in function between the two wheat *cdc2*-like genes, I compared their amino acid sequences in the regions identified as functionally important by the mutational evidence presented above (see Fig. 4.10). Almost all important amino acid residues are present in both *cdc2TaA* and *cdc2TaB* proteins, except three residues. One is at position 112, where the *cdc28-9* mutation demonstrated that Tyr cannot replace Cys, however, both wheat *cdc2*-like proteins have a Leu in this position, and *S. pombe cdc2* also has a different residue (Val) in this position. All other plant *cdc2* homologues have a Leu in this position too. The second position is at 139, where positive mutants *cdc2-1w/2w* occurred by substitution of Asp for Gly and again both wheat *cdc2*-like proteins (as well as other plant *cdc2* homologues, see Fig. 3.12) have the same substitution of Thr in this position, providing no evidence for difference in function of the two wheat Cdc2-like proteins.

The third position at which the two wheat *cdc2*-like proteins differ is at 277, where Cdc2TaA has Thr and Cdc2TaB has Asn. Assessing this evidence is made uncertain because of ambiguity concerning phosphorylation of Ser277. In chicken phosphorylation of this amino acid has been described as involved in the regulation of Cdc2 activity in G1 phase (Krek and Nigg, 1991) but this phosphorylation has not been confirmed in other animals. It may be significant that most plant Cdc2 proteins substitute for serine another amino acid that can be phosphorylated, which is threonine. The two known plant Cdc2 molecules that substitute a non-phosphorylateable amino acid at this position are *Arabidopsis cdc2a* and rice *cdc2Os-2*, which both have Asn. It may be significant that neither of these complements *cdc28*^{ts} (*cdc28-1*^{ts}, *cdc28-13*^{ts}, *cdc28-4*^{ts}) well; Ara *cdc2a* does so partially (Ferreira et al., 1991) and *cdc2Os-2* not at all (Hashimoto et al., 1992). It is consistent with this pattern that wheat *cdc2TaA* has Thr, like alfalfa *cdc2* and soybean *cdc2* (Fig. 3.12), and can complement G1-S and G2-M deficiencies (Fig. 4.3) as do alfalfa and soybean (Hirt et al., 1991; 1993; Miao et al., 1993), whereas wheat

cdc2TaB has Asn and cannot complement any of the three *cdc28^{ts}* alleles used in the present study. These correlations appear to indicate that phosphorylation of the amino acid at 277 (adjacent of the perfectly conserved RI doublet) could be important in yeast and in some *cdc2*-like plant proteins. Direct evidence is not available since only in chicken has phosphorylation been detected in this region, however if only a small proportion of *cdc2* proteins need to active at G1-S the levels of this phosphorylation maybe extremely difficult to detect.

A noteworthy phenomenon in complementation test by *cdc2TaA* was that there was a lag period (the first 12 h when shifting to the restrictive temperature) before rescue of *cdc28^{ts}* mutants was detectable (Fig. 4.5). It is possible that this indicates that translation of *cdc2TaA* protein is slow, although there was no direct evidence. The GC-rich leading sequence before the putative ATG start codon of both wheat *cdc2*-like genes (see chapter 3) may encumber the translation since in yeast, most mRNAs have AT-rich leader sequences (Kozak 1989). Poor translation could contribute to incomplete complementation of *cdc28-13^{ts}* and *cdc28-1N^{ts}* by *cdc2TaA* and the inability to complement *cdc28^{ts}* mutants shown by *cdc2TaB*. If this inference is true, the complementation could be improved by removing the GC-rich leading sequence when subcloning of wheat *cdc2*-like cDNAs into yeast expression vector. However Western blotting did confirm the presence of at least some expressed protein from both Cdc2TaA and Cdc2TaB (Fig. 4.9).

Care was taken to establish that the genes were expressed in the yeast cells. A fortuitous slight difference in electrophoretic mobility allowed the plant and yeast *cdc2* proteins to be resolved, and it was observed that antibody raised against the plant carboxy-terminal peptide allowed the plant protein to be specifically detected (Fig. 4.9A), while antibody against the PSTAIR sequence shared by the plant and yeast proteins detected both plant and yeast proteins (Fig. 4.9B). Levels of expression of plant proteins in yeast have never been checked in previous studies of complementation by plant *cdc2*-like genes. Thus failure to express or accumulate significant levels of the protein may have been the explanation for some failure of complementation, such as *Arabidopsis cdc2a* (Ferreira et al., 1991) and rice *cdc2Os-2* (Hashimoto et al., 1992). In

the present study a lower level of Cdc2TaB protein was detected by antibody against its C-terminal. This antibody was seen to detect both Cdc2TaA and Cdc2TaB (Fig. 4.9). Given that Cdc2/Cdc28 protein is not limiting in proliferating yeast cells, from evidence that increased expression of *cdc2* does not accelerate entry into division (Durkacz et al., 1986) and also that $p34^{cdc2}$ is recruited into active complexes from a large pool of inactive monomer (Murray and Hunt, 1993) it is unlikely that slightly reduced levels of Cdc2TaB protein would result in complete failure to complement; rather an inability of the plant protein to perform all of the essential functions that have been lost from the three $cdc28^{ts}$ mutants, is indicated.

The ability of a foreign gene to complement $cdc2^{ts}/cdc28^{ts}$ mutants can indicate functional homology, however, failure to complement $cdc2^{ts}/cdc28^{ts}$ mutants cannot be taken as proof that in the cell of origin the gene under test does not function as *cdc2*. Of particular interest is the possibility that plants contain a variant of $p34^{cdc2}$ specialised for activity at G1-S. If Cdc2TaB only acts at G1-S phase, it may not be able to complement $cdc2^{ts}/cdc28^{ts}$ mutants. For example in *Xenopus*, a *cdc2* related gene *Eg1* (65.3% identity to *S. pombe cdc2*, later designed as *cdk2*), which only functions at the G1-S transition (Fang and Newport, 1991), could not complement $cdc2^{ts}/cdc28^{ts}$ mutants (Paris et al., 1991) although other *cdk2* genes have been found to complement. The human *cdk2* gene has given different results in complementation test by two research groups. Elledge and Spottswood (1991) found it could complement the $cdc28\text{-}4^{ts}$ mutant only if a second mutation occurred. While Ninomiya-Tsuji et al. (1991) found it could complement the $cdc28\text{-}13^{ts}$ mutant with no evidence that additional mutation of the yeast enzyme occurred and could even complement a null *cdc28* mutant. Thus, while complementation does not depend upon the subjective assessment of degrees of sequence similarity to evaluate possible homology, it is a technique that has its own uncertainties. The situation with respect to possible G1-S active CDKs in plants will be greatly clarified when it is possible to develop antibodies against plant cyclins to recover immunoprecipitates that can reveal what CDKs are in complex and enzymically active in particular cycle phases.

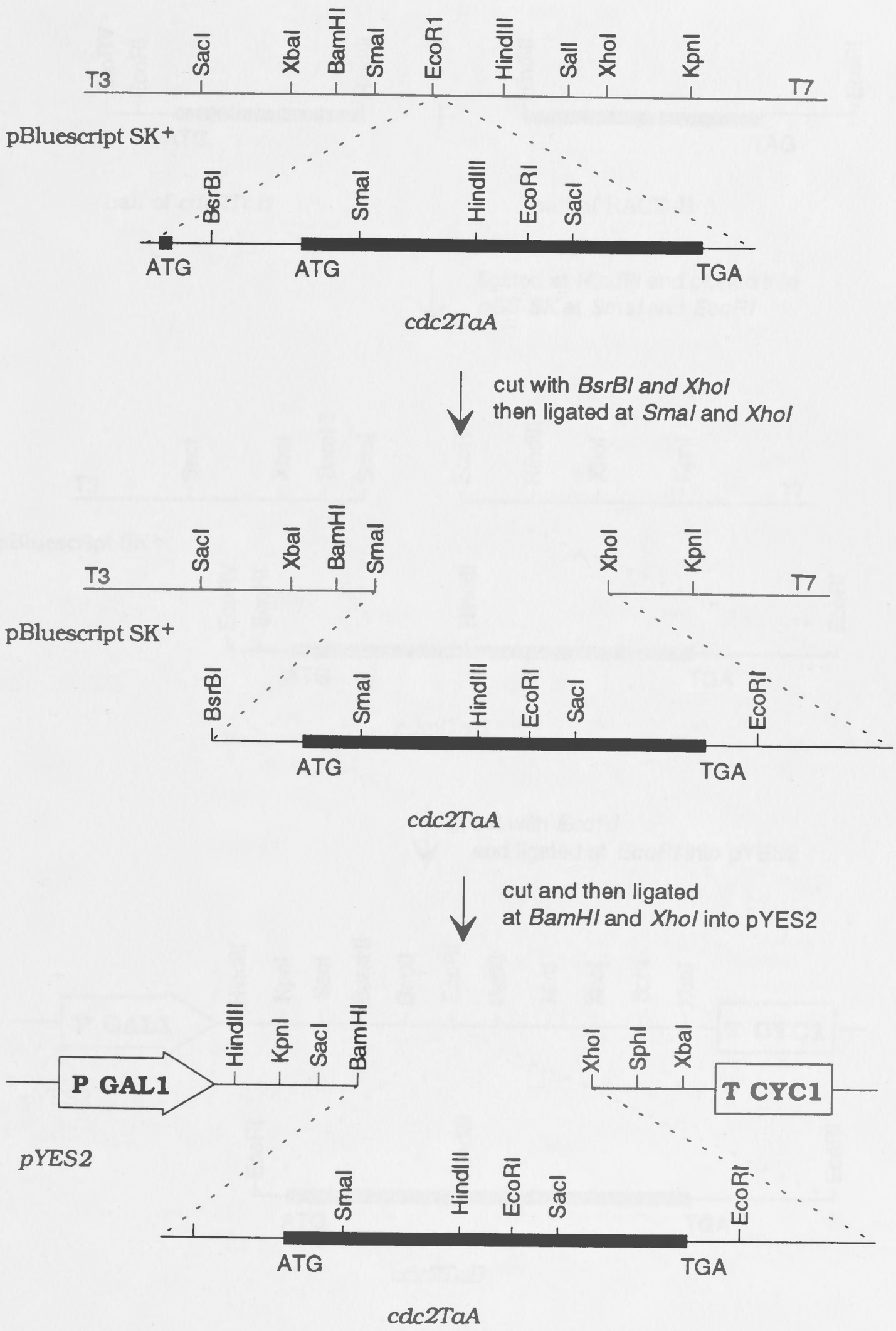


Fig. 4.1 Subcloning of*cdc2TaA* cDNA into yeast expression vector pYES2. Broad black lines indicate Open-Reading-Frame of*cdc2TaA*.

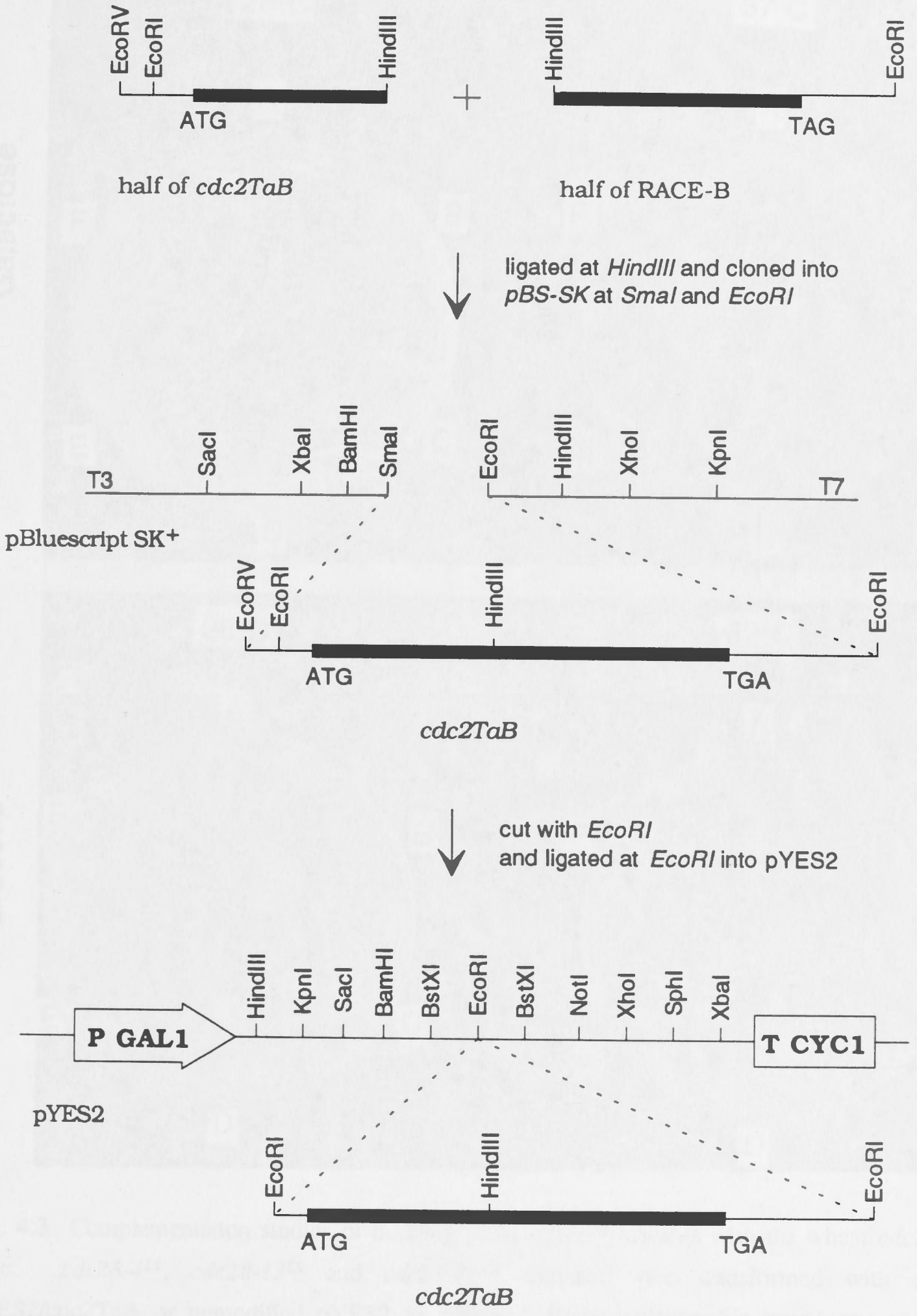


Fig. 4.2 Subcloning of*cdc2TaB*cDNA intoyeast expression vectorpYES2. Broad black lines indicate Open-Reading-Frame of*cdc2TaB*

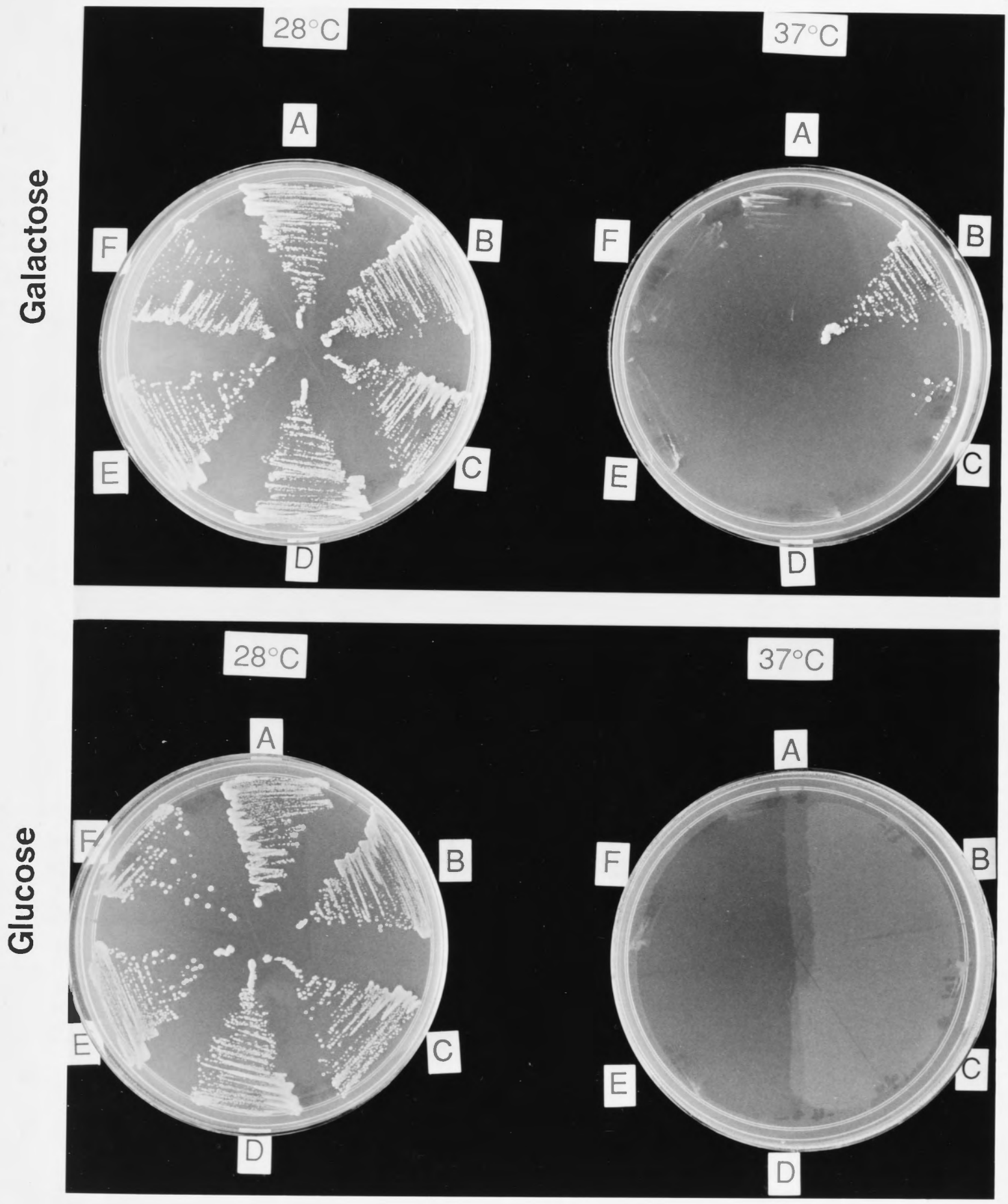


Fig. 4.3 Complementation studies of budding yeast *cdc28*^{ts} mutants with the wheat *cdc2TaA* gene. *cdc28-4*^{ts}, *cdc28-13*^{ts} and *cdc28-1N*^{ts} mutants were transformed with either pYES2/cdc2TaA or unmodified pYES2 as control. Uracil prototrophic transformants were selected and plated onto galactose (upper) and glucose medium (lower), then grown for 4 days at 28°C or at 37°C.

A: *cdc28-4*^{ts} cells transformed with pYES2/cdc2TaA;

B: *cdc28-13*^{ts} cells transformed with pYES2/cdc2TaA;

C: *cdc28-1N*^{ts} cells transformed with pYES2/cdc2TaA;

D: *cdc28-4*^{ts} cells transformed with pYES2;

E: *cdc28-13*^{ts} cells transformed with pYES2;

F: *cdc28-1N*^{ts} cells transformed with pYES2.

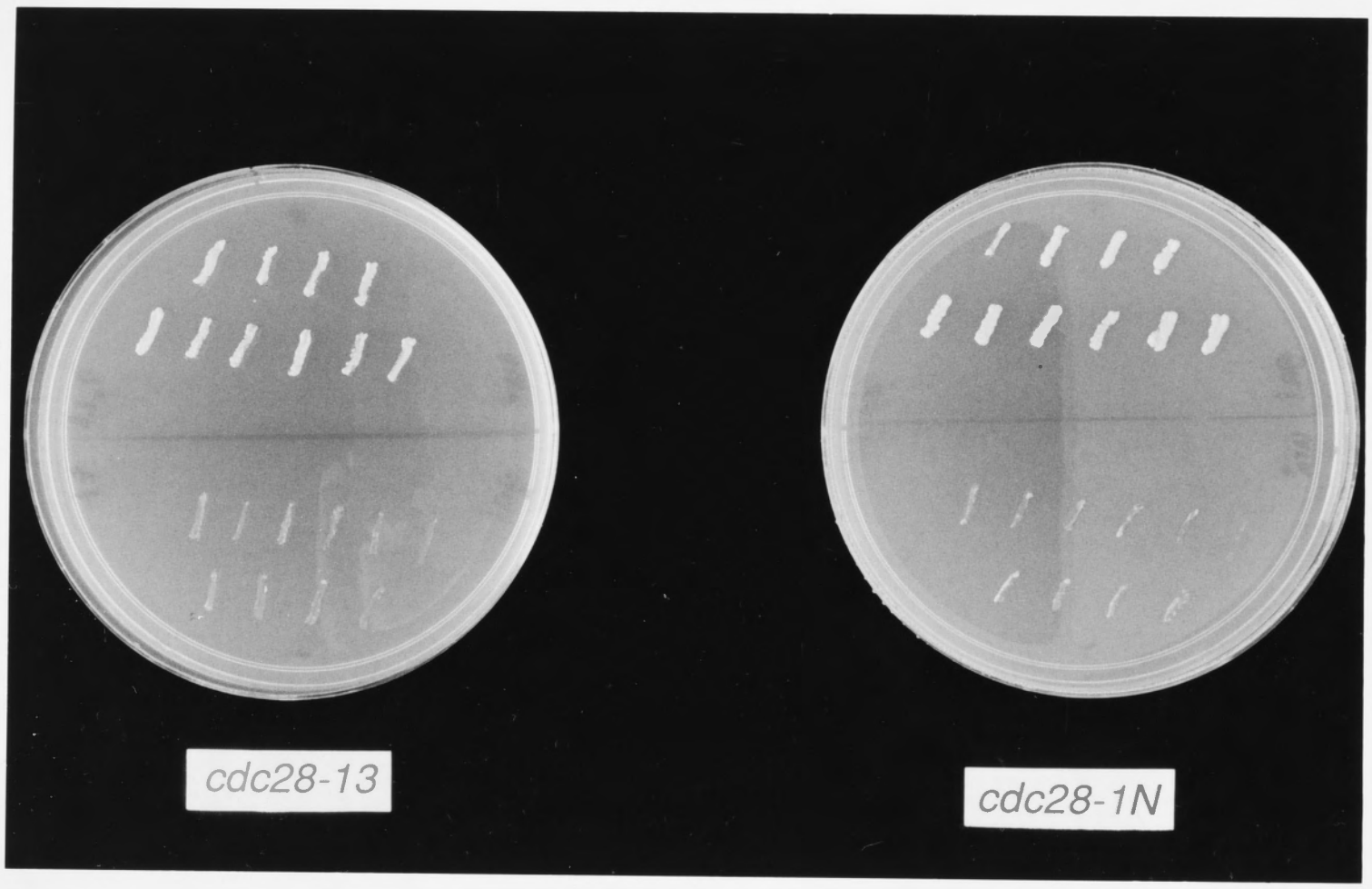


Fig. 4.4 Mutants *cdc28-13*^{ts} and *cdc28-1N*^{ts} carrying pYES2/cdc2TaA (upper half of each plate) or having lost pYES2/cdc2TaA (lower half of each plate) grown on uracil + galactose plates at 37°C for 2 days. Cells carrying pYES2/cdc2TaA proliferated but cells without pYES2/cdc2TaA did not.

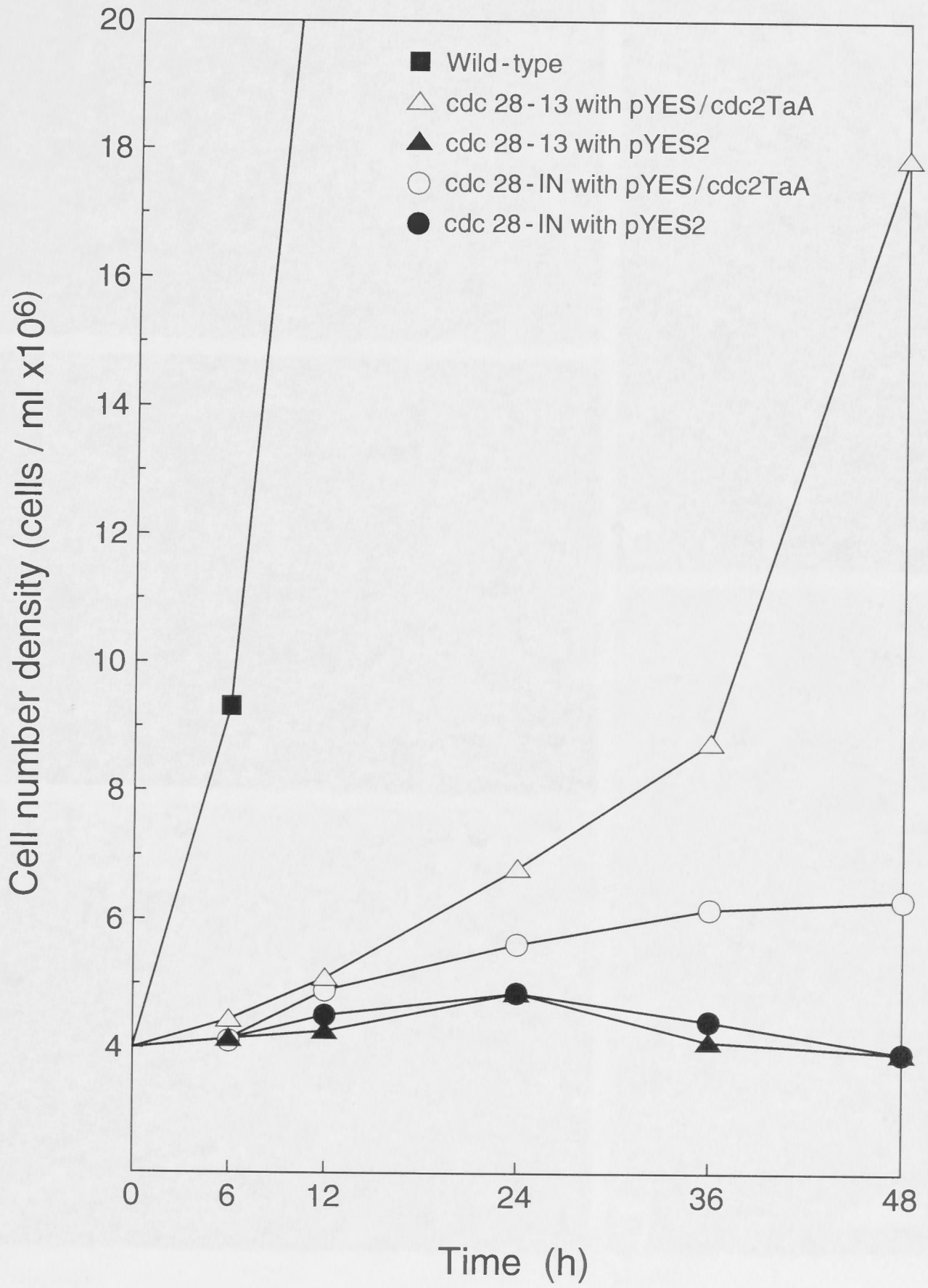


Fig. 4.5 Growth curve of mutant cells and wild - type w303 cells cultured in galactose medium at 37°C.
0 h was the time cells transferred from glucose medium at 28°C to galactose medium at 37°C.

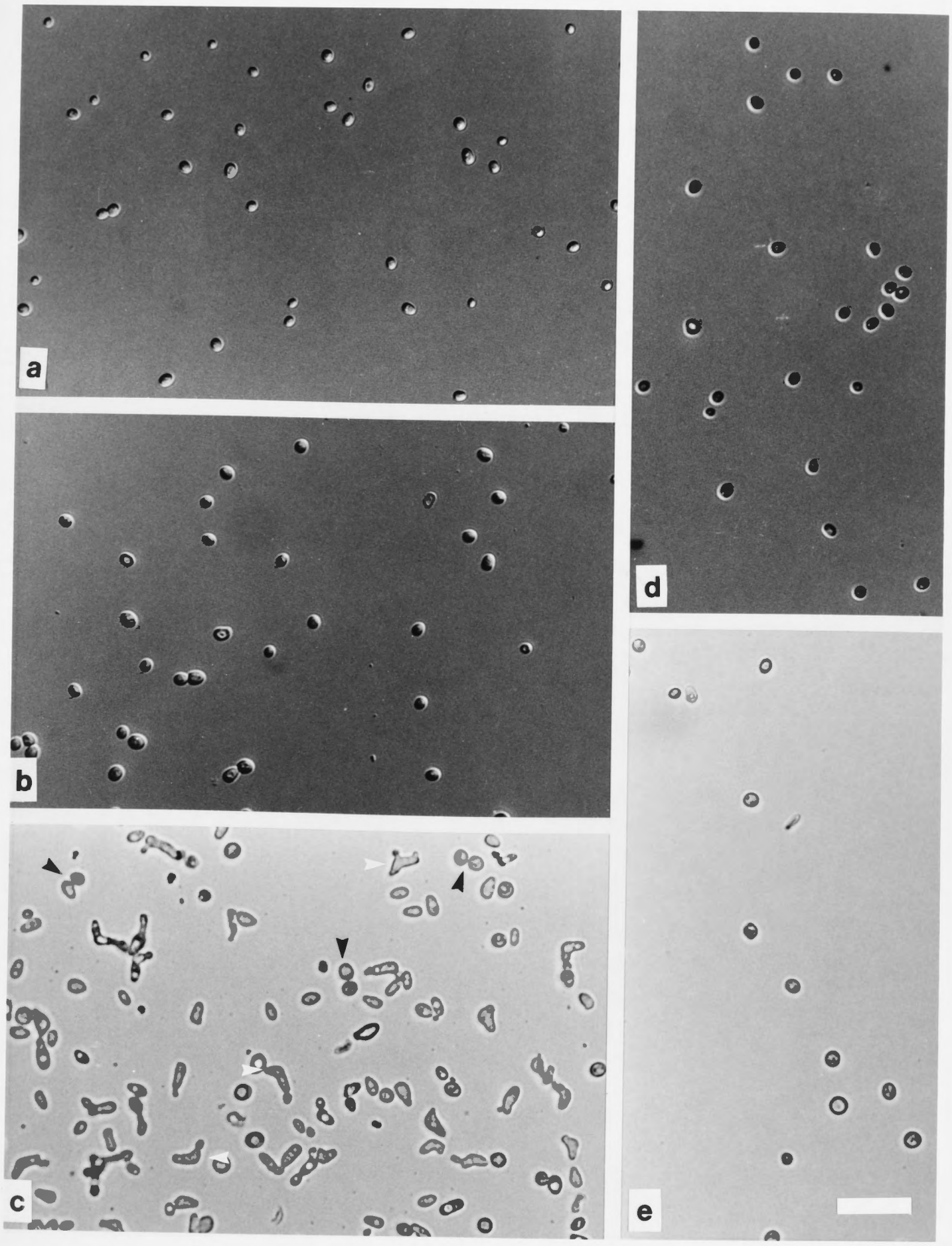


Fig. 4.6 Morphology of *cdc28-13^{ts}* cells carrying pYES2/cdc2TaA or unmodified pYES2, grown in liquid galactose medium at 37°C.

a, **b**, and **c**: cells carrying pYES2/cdc2TaA at 0 h (a, immediately after shifting cells from glucose medium at 28°C to galactose at 37°C), and after 12 h (b) and 48 h (c) at 37°C, respectively.

d and **e**: cells carrying pYES2 at 0 h and after 48 h at 37°C, respectively.

Black arrows show dividing cells and white arrows show the aberrant morphology of cells with an elongated bud or multiple buds, indicating that the *cdc2TaA* protein did not allow division to fully keep pace with growth. The cells carrying pYES2 remained unbudded and large (e) indicating a blocked in G1 phase.

All pictures are at the same magnification. Scale bar, 8 μm.

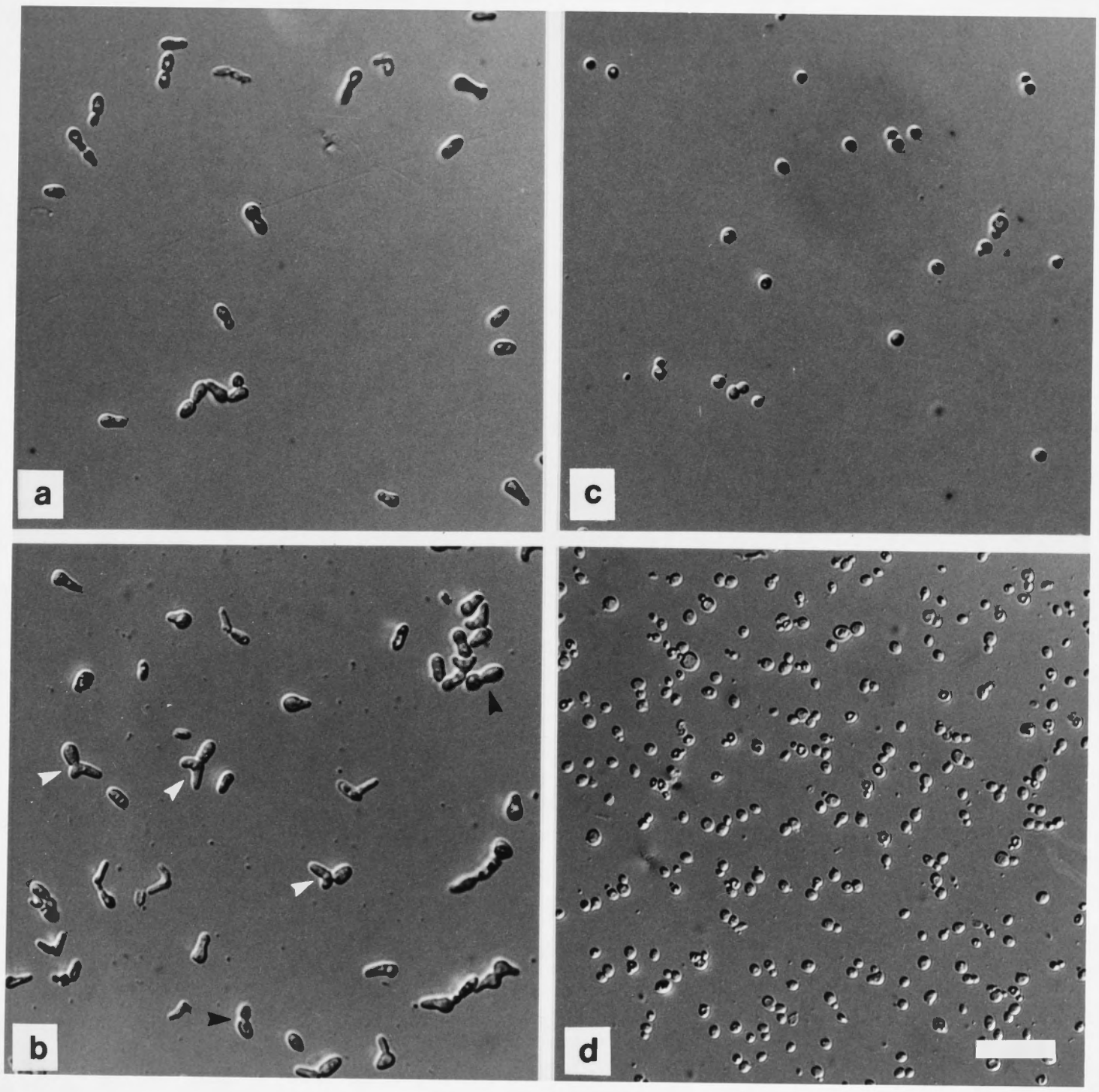


Fig. 4.7 Morphology of *cdc28-1N^{ts}* cells carrying pYES2/cdc2TaA grown in liquid galactose medium at 37°C, and wild-type cells grown in the same conditions.

a and **b**: *cdc28-1N^{ts}* cells carrying pYES2/cdc2TaA at 0 h (when cells had just been shifted from glucose medium at 28°C to galactose medium at 37°C) and after 12 h at 37°C.

c and **d**: wild-type cells at 0 h and after 12 h at 37°C.

Black arrows show the mutant cells that were dividing at the restrictive temperature; white arrows show some cells with aberrant morphology. The high proportion of budded cells in **a** and **b** is consistent with the ability of uncomplemented cells to complete START and indicates that progress through mitosis is slower than normal when complemented by *cdc2TaA*.

All pictures are at the same magnification. Scale bar, 8 μm.

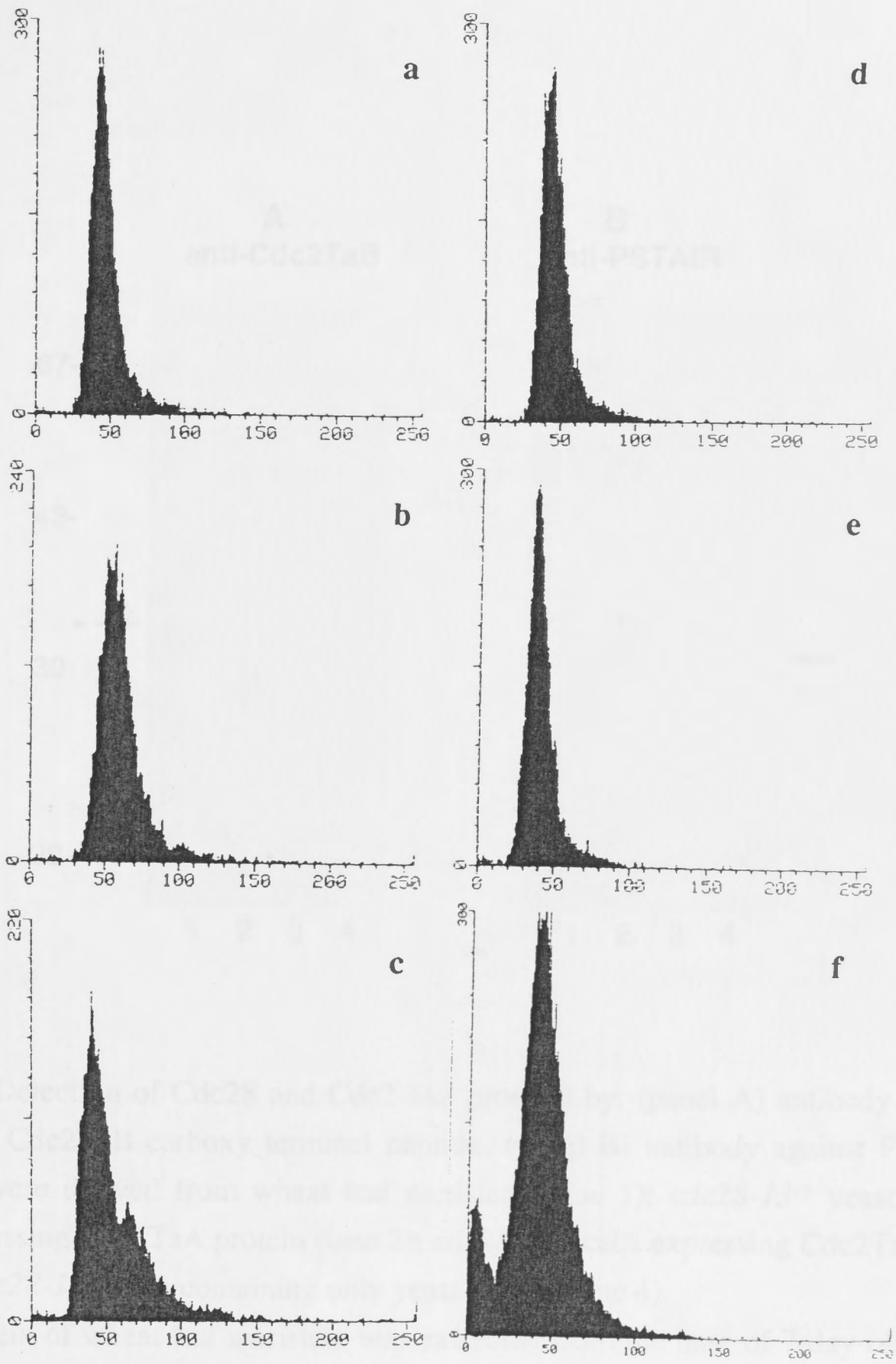


Fig. 4.8 Flow cytometric analysis of propidium iodide-stained *cdc28-13^{ts}* cells, carrying pYES2/cdc2TaA (**a**, **b**, **c**), or carrying unmodified pYES2 (**d**, **e**, **f**), after shift from glucose medium at 28°C to galactose medium at 37°C for 0 h (**a**, **d**), 12 h (**b**, **e**), 48 h (**c**, **f**).

The ordinate for each plot represents frequency while the abscissa represents relative fluorescence intensity.

The peak at about 40 fluorescence units indicates cells in G1 phase with 1C DNA content. The small peak at about 70-80 in **c** indicates cells in G2 and M phases with 2C DNA content; it arises because complementation by the *cdc2TaA* gene allowed *cdc28-13^{ts}* cells to pass the G1-S transition at the restriction temperature. While control cells carrying unmodified pYES2 did not restore the ability of DNA replication and division (**d**, **e**). The small peak in **f** at 0-30 fluorescence units indicates dead cells.

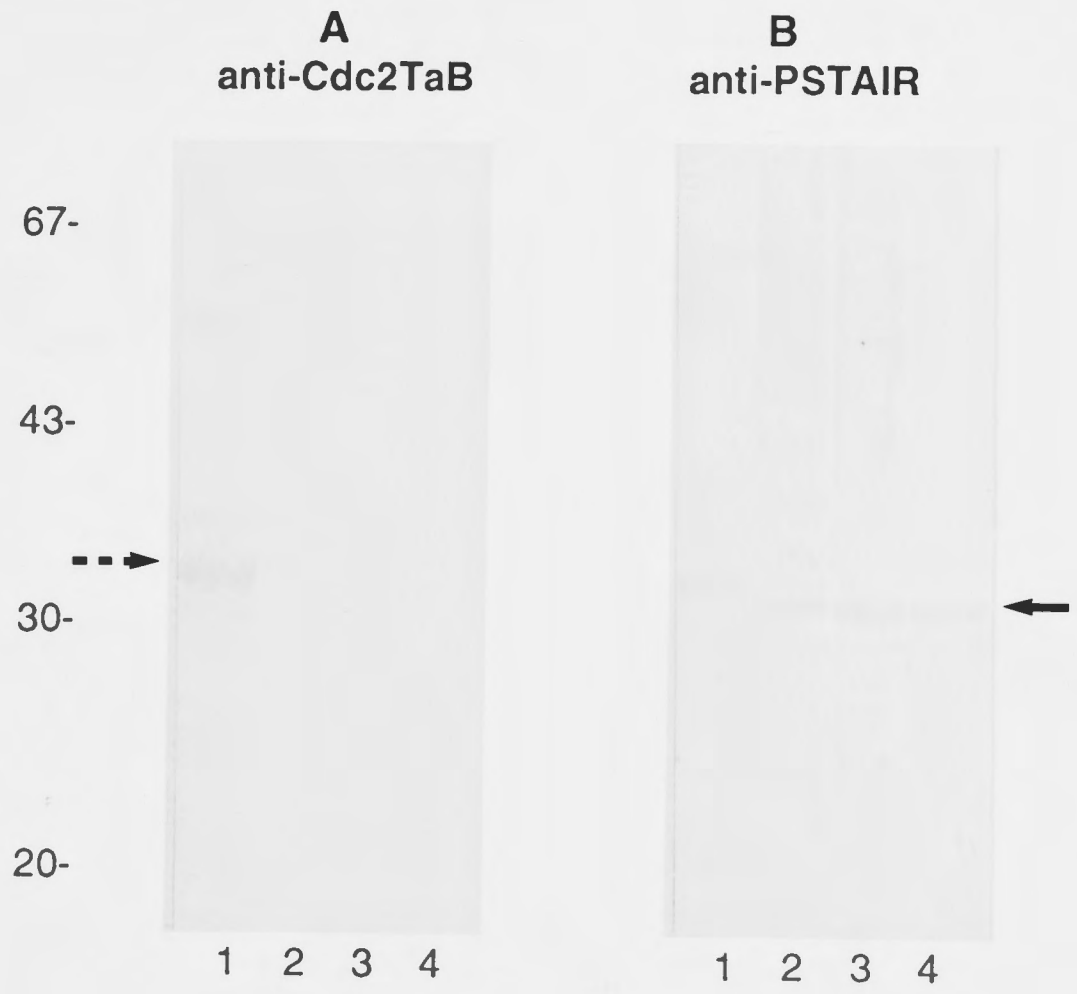


Fig. 4.9 Detection of Cdc28 and Cdc2-like proteins by; (panel A) antibody specific for wheat Cdc2TaB carboxy terminal peptide, (panel B) antibody against PSTAIR. Proteins were derived from wheat leaf meristem (lane 1); *cdc28-13^{ts}* yeast mutant cells expressing Cdc2TaA protein (lane 2); *cdc28-13^{ts}* cells expressing Cdc2TaB (lane 3); and *cdc28-13^{ts}* cells containing only yeast Cdc28 (lane 4).

Total protein of wheat leaf meristem was extracted from the base of 7-day-old leaves (see Chapter 2, 2.2.3.1). Yeast proteins were extracted from cells after culture in liquid medium supplemented with galactose at 28 °C for two days (see Chapter 2, 2.2.14.6). Each lane carried 50 µg of total protein.

Blot A was immuno-probed with rabbit antiserum raised against C-terminal peptide of Cdc2TaB protein, which detected both Cdc2TaA and Cdc2TaB (- ►), but not yeast Cdc28 protein (◄-).

Blot B was immuno-probed with anti-PSTAIR antibody, which detected both wheat Cdc2-like proteins and yeast Cdc28 protein. The plant Cdc2-like protein ran a little more slowly than yeast Cdc28 protein and is seen faintly in lanes 2 and 3 above the more abundant Cdc28 protein.

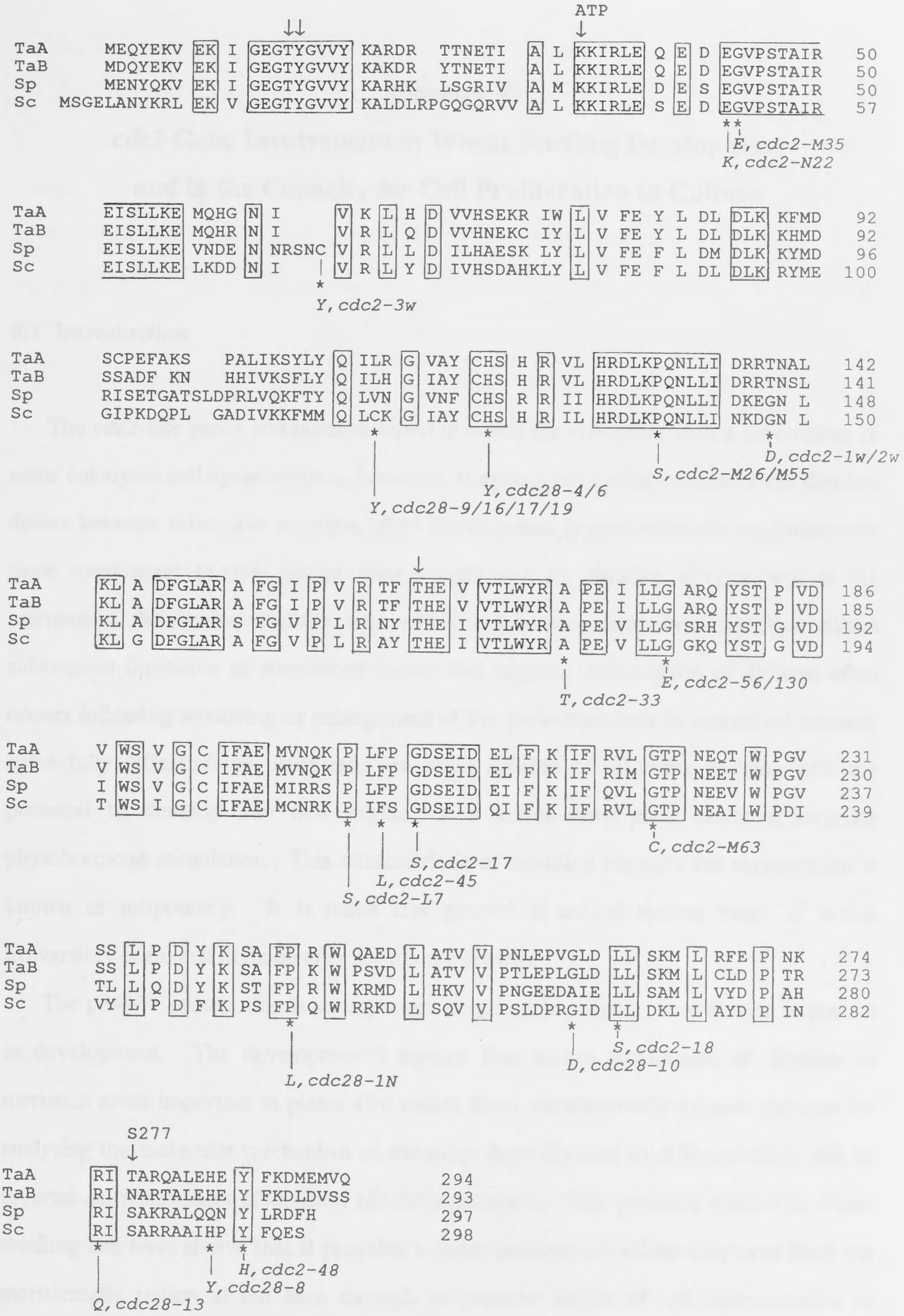


Fig. 4.10 19 mutation points of *cdc2*^{ts} and *cdc28*^{ts} marked with * (ref: Carr et al., 1989; MacNeill et al., 1991; Lorincz and Reed, 1986). Arrows indicate amino acids whose phosphorylation/ dephosphorylation is significant in Cdc2 function. Boxes indicate conserved regions, as in Fig. 3.12.

Chapter 5

cdc2 Gene Involvement in Wheat Seedling Development and in the Capacity for Cell Proliferation in Culture

5.1 Introduction

The *cdc2*-like genes and proteins found in wheat are consistent with a universality of some eukaryote cell cycle controls, however, temporal and spatial control of cell division differs between taxa. For example, plant development is predominantly postembryonic since most plant growth occurs after germination by iterative development at the meristems. Meristems produce cells with the frequency and orientation that allows subsequent formation of specialised tissues and organs. Resumption of division often occurs following wounding or enlargement of the growth regions in successive seasons. Even fully differentiated plant cells are often capable of resuming division with the potential to develop into new organs or a whole new plant under appropriate phytohormone stimulation. This characteristic of retaining capacity for regeneration is known as totipotency. It is much less general in animal tissues many of which irreversibly cease cell division after embryonic growth.

The p34^{cdc2} protein that is so important in cell cycle progress may also be important in development. The developmental pattern that makes localisation of division to meristem areas important in plants also makes them experimentally suitable subjects for analysing the molecular mechanism of transition from division to differentiation, and its reversal on resumption of division (de-differentiation). Our previous studies in wheat seedling leaf have shown that it provides a linear gradient of cell development from the meristematic region at the base through progressive stages of cell differentiation to mature photosynthetic cells towards the tip. p34^{cdc2}-like protein detected by PSTAIR antibody has been found at high levels only in meristem tissue and is at low levels in differentiated tissue (John et al., 1990). Differentiation is accompanied by 15-fold

decline in the level of p34^{cdc2}-like protein relative to other proteins and this has led to the proposition that the decline plays a part in enforcing abstention from division (John et al., 1990). The distribution of active p34^{cdc2} rather than of the enzyme protein is likely to bear a more direct relationship to cell division activity. Therefore in this chapter, I measured p34^{cdc2}-like kinase activity during the development of cells in the wheat seedling leaf and found that high activity of p34^{cdc2} kinase was restricted to the active division region of the leaf meristem and drops even more sharply during the switch to cell differentiation than does the amount of the catalytic subunit p34^{cdc2} measured as the total amount of PSTAIR-reacting 34 kDa protein.

Monocotyledonous plants, such as wheat, are interesting subjects for study of division control because their capacity for totipotency is restricted to meristems. Differentiated cells have been found difficult or impossible to stimulate into resumption of division (reviewed by Vasil, 1994) and this difficulty has implications for the use of transgenic techniques in the breeding of the cereals for food. The recalcitrance of monocotyledonous cells to resumption of division is in sharp contrast to what has been observed in many dicotyledonous plants. I therefore studied the changes of p34^{cdc2} protein level and kinase activity during the *in vitro* culture of wheat seedling leaf segments in the presence of the auxin analogue, 2,4-D, which has been reported to support cell division in cultured cereal tissue (Wernicke et al., 1986).

5.2 Results

5.2.1 Cell division activity in wheat seedling leaf

The first leaves of 7-day-old wheat seedlings, which were about 80-90 mm long, were isolated by taking off the coleoptile then excising the first leaf and discarding the second leaf that was developing inside it. These first seedling leaves provide a linear developmental gradient extending upwards to the tip, from a basal region of active cell division, through a series of progressively differentiating leaf blade cells, to a zone of mature photosynthetic cells (Boffey et al., 1979; Wernicke et al., 1986; John et al.,

1990). From such leaves successive 2 mm segments were cut for analysis of current cell division and capacity for division in culture. Determination of mitotic index (Method 2.2.2) showed that cell division was restricted to the meristem occupying the region 0-10 mm from the leaf base (Fig. 5.1). The first 2 mm of the leaf had the highest percentage of mitotic cells and decreasing mitotic activity was found in succeeding segments towards the tip. Cells in the first three segments (0-6 mm) were small and cells that had ceased dividing gradually increased in size, predominantly by elongation in the direction of the long axis of the leaf, in the following samples. In the sixth segment (10-12 mm) division was almost absent, being restricted to the specialised divisions that form stomata, with the result that the percentage of mitotic cells in this region was about 1/2000. No cells were detected in mitosis in the seventh and eighth segments (12-16 mm).

The distribution of mitotic activity that I observed is consistent with earlier reports (Wernicke et al., 1986; Wernicke and Milkovits, 1987; John et al., 1990). My observations of the level and activity of p34^{cdc2}-like protein may therefore be compared with the earlier studies in which the enzyme was not assayed.

5.2.2 p34^{cdc2} protein level and kinase activity in wheat seedling leaf

John and his colleagues have earlier reported that the p34^{cdc2}-like protein of seedling wheat leaf accumulated to a maximum level in dividing cells and accumulation ceased during subsequent cell growth and expansion, resulting in a decline to about one-fifteenth in level relative to other proteins (John et al., 1990). In this earlier study the enzyme activity of the protein was not measured, therefore to investigate how p34^{cdc2} kinase activity is regulated during development of the seedling leaf, the same segments of seedling leaves as were analysed earlier (John et al., 1990) were taken for assay of the p34^{cdc2} kinase activity (see Fig. 5.3).

For assay of p34^{cdc2}-like H1 histone kinase purification with p13^{suc1} covalently coupled to agarose beads (cyanogen bromide Sepharose) was employed (Brizuela et al, 1987). The usefulness of this reagent for analysis of plant proteins has been established

by John, Sek and Hayles (1991) who showed that other protein kinases are present in wheat leaf extracts but can be discarded by p13^{suc1} chromatography. The proteins with specific affinity for p13^{suc1} were recovered by first depleting proteins that might have affinity for polysaccharide by reaction with agarose beads, then binding to beads carrying covalently coupled p13^{suc1}, then extensive washing in buffer with detergent and salt and finally elution of proteins with p13^{suc1} solution (Methods in 2.2.5.2). The requirements for quantitative recovery of p34^{cdc2} kinase were investigated by determining the recovery of p34^{cdc2}-like kinase using increasing amounts of p13^{suc1}-beads and also determining the requirement for p13^{suc1} concentration in solution to fully elute p34^{cdc2}-like kinase that was bound to p13^{suc1} on beads (Fig. 5.2).

The activity of p34^{cdc2}-like H1 histone kinase recovered in the p13^{suc1} fraction was sharply restricted to the meristem region, mainly at 0-8 mm (Fig. 5.3). The highest activity was detected in the first segment (0-4 mm), where cells were dividing most actively, with 2.8-4.6% in mitosis (Fig. 5.1). Activity declined steeply to one fifth and one ninth in the second and third segment (4-12 mm), where the mitotic index also dropped proportionately to 0.26-1.5%. Only very little p34^{cdc2} kinase activity was detected in the fourth segment (12-16 mm), where the mitotic index was 0, and there was essentially zero p34^{cdc2} kinase activity from the fifth segment (16-20 mm) to the leaf tip (Fig. 5.3). Therefore p34^{cdc2}-like H1 histone kinase activity precisely predicts where mitotic activity occurs in the leaf and does so more precisely than the level of p34^{cdc2}-like protein, which declines less sharply (Fig. 5.4) as will be discussed.

To test whether the decrease of p34^{cdc2}-like H1 histone kinase activity in the extracts from nonmeristematic leaf tissues was perhaps due to accumulation of diffusible non-specific inhibitory metabolites that might arise from the specialised metabolism of differentiating cells, extracts from different leaf regions were mixed. If there are enzyme-inactivating chemicals present in the vacuoles of mature cells, the grinding and extracting of proteins from these tissues could release these inhibitors, however their presence could be detected by their capacity to reduce the enzyme activity recovered from mixtures of tissues. The extracted p34^{cdc2} kinase activity from mixtures of meristematic (segment 1, 0-4 mm) and non-meristematic tissue (segments 5 and 10, 16-20 mm and

70-74 mm) (Fig. 5.5) indicated no significant decrease in activity due to possible inhibitors in mature cells. The tissues were mixed after grinding to powder in liquid nitrogen and before the addition of extraction buffer (NDE buffer, section 2.2.5.2), therefore inhibitors potentially present in mature cells would have had full access to the active enzyme of meristematic cells. It can be concluded that the lower activity of p34^{cdc2}-like H1 histone kinase in mature cells is inherent in the enzyme protein and, by analogy with other cell types, may be due to inhibitory phosphorylation or lack of cyclin subunits.

5.2.3 *In vitro* culture of wheat seedling tissue

To test for a possible correlation between presence of active p34^{cdc2} kinase and capacity for continued proliferation *in vitro*, excised 4 mm segments of first seedling leaf (as used in p34^{cdc2} kinase assay), were cultured on MS agar supplemented with 2,4-D at concentrations from 0.6 μM to 160 μM. After 2-3 weeks of culture, low concentrations of 2,4-D had induced formation of callus and roots in the first segment (0-4 mm, Fig. 5.6). At the same 2,4-D concentrations cells in the second and third segments (4-12 mm) elongated without resuming division, whereas the distal segments all retained the length at time of excision, indicating that fully expanded cells neither expanded further nor divided on transfer to auxin-containing medium. At intermediate concentrations of 2,4-D, the first three segments (0-12 mm) were able to respond to auxin by forming callus while segments beyond 12 mm were unable to respond (Fig. 5.6). Although the intermediate concentrations of auxin were able to induce proliferation in the region 4-12 mm, which did not respond at the lower auxin concentrations, the proliferation was not sufficiently organised to form roots.

To further study the ability of tissue to respond to auxin in culture and its possible relationship with p34^{cdc2}, smaller segments (2 mm long) were excised from first seedling leaf base in the region 0-24 mm and cultured on MS agar supplemented with 2,4-D at 0, 0.5, 2.5, 10, 50, 100 and 150 μM. After 3 weeks of culture in 0.5 μM 2,4-D the first segment was able to respond by forming callus and regenerating roots (Table 5.1 and

Fig. 5.7). At intermediate to high concentrations of 2,4-D (50-150 μM), the maximum response to auxin was found at 10 mm above the base, but higher concentrations of auxin became toxic resulting in browning at the margins of callus (Table 5.1). The toxicity of higher concentrations of auxin to cells that were already dividing is of interest and may suggest that division is linked with high responsiveness to auxin, so that only cells that are ceasing to divide and perhaps diminishing in responsiveness, as at 10 mm above the base, can tolerate 50-150 μM 2,4-D.

To investigate the structural relationship of normal divisions and those induced by high concentrations of 2,4-D in culture, meristematic tissue (Segment 1) was examined after staining with acetocarmine when freshly excised from the first seedling leaf and also after 3 weeks of culture. Fig. 5.8 (b) shows that there was no continuation of cell division in the segment cultured on MS agar without auxin although the segment elongated due to the expansion of the original meristem cells which became vacuolated in culture. Cell elongation is not therefore dependent upon exogenous hormone in this tissue. In the 2,4-D treated callus, cell division was detected but showed loss of the regular cell positioning found in the intact leaf base (Fig. 5.8 a). The division rate in callus was also lower than in the meristem (Fig. 5.8 c, d). Therefore content and activity of p34^{cdc2} kinase was assayed in freshly excised and also cultured meristem tissue.

5.2.4 p34^{cdc2} protein level and kinase activity in leaf tissue before and after culture

The p34^{cdc2} level and kinase activity in leaf segments before culture are shown in Fig. 5.9 (a) and Fig. 5.11 (a). After culture for three weeks on MS medium with 50 μM 2,4-D, the level of p34^{cdc2}-like protein had declined throughout (Fig. 5.9 b), while the p34^{cdc2}-like kinase activity had declined only in the first two segments (0-4 mm) but increased in the third, fourth and fifth segment (4-10 mm) based on assay of the enzyme from the same amount of extracted total protein (Fig. 5.10 b and Fig. 5.11 d). A striking feature of the comparison was the extent to which p34^{cdc2}-like protein declined in the region 4-10 mm from levels present at excision (Fig. 5.9) although the cells showed an increase in extent of activity of p34^{cdc2} (Fig. 5.11 d) due to auxin stimulation compared

with activity at time of excision (Fig. 5.11 a). The formation of callus involved a rate of cell division (mitotic index 1%, Fig. 5.8) higher than that prevailing in the region 6-15 mm before excision (Fig. 5.1), but lower than that in the region 0-6 mm before excision (Fig. 5.1). In the region 0-8 mm the protein declined to about half relative to total extracted protein and at the margins of the region, although cells were able to respond to 2,4-D by continuing division, the decline was more extensive; to 15% in the region 8-12 mm and to less than 10% in the region 12 to 20 mm. Retention of the p34^{cdc2} protein therefore depended upon some continuation of cell division in the region 0-8 mm but the lower rate of division, with a mitotic index of about 1% (Fig. 5.8) correlated with lower levels of the enzyme than in the meristem of the intact seedling leaf. The considerable drop in p34^{cdc2}-like enzyme protein in the regions distal to 10 mm could indicate that the enzyme inherited from the time of earlier cell division is subject to breakdown under prolonged culture without cell division. Certainly the regions in which appreciable amounts of p34^{cdc2}-like protein could be recovered after culture (Fig. 5.9 b) were those in which active enzyme could be found (Fig. 5.10 and 5.11 d) and even an increased activity found in the 4-10 mm region. Unlike freshly excised tissue which, in the region 10-20 mm (Fig. 5.4) contained appreciable amounts of p34^{cdc2}-like protein that was not active, tissue from the 4-10 mm region had lost much of its p34^{cdc2}-like protein after culture, although the residual enzyme had become more activated due to auxin stimulation than it was at time of excision.

The most significant feature of the comparison between p34^{cdc2} level and activity at time of excision with behaviour of the tissue in culture, was the close correlation at lower 2,4-D concentration (0.5 μM) between presence of active p34^{cdc2}-like enzyme at excision and capacity to form callus in culture. The maximum concentration of 2,4-D that could be used to form healthy (non-browning) callus was 50 μM and the most distal tissue capable of responding was that between 0-10 mm from the leaf base (Table 5.1). Exactly this tissue was found to contain active p34^{cdc2} at time of excision (Fig. 5.3 and 5.4) although activity levels were low in the region 4-10 mm. Furthermore there was no evidence that 2,4-D was able to stimulate synthesis of p34^{cdc2}. Even at concentrations so strong that root formation was disrupted, such as 50 μM, the hormone was only able

to stimulate retention of part of the enzyme present at excision. In the region 8-12 mm 85% of the p34^{cdc2}-like protein was lost (Fig. 5.9) although the residual level was able to support slow proliferation forming callus (segment 5, Table 5.1 and Fig. 5.7) and this correlated with detectable enzyme activity (Fig. 5.10). It was revealed that low levels of p34^{cdc2} activity were adequate to support slow callus formation seen in segment 1 after culture on 0.5 μM 2,4-D (Fig. 5.11 c). Under these conditions the segment was eventually able to establish organised division giving root primordia (Fig. 5.7) in which localised high concentrations of p34^{cdc2} are predicted.

5.3 Discussion

The data presented in this Chapter support the idea that accumulation of the *cdc2* gene product is controlled in plant development and may contribute to the localisation of division. p34^{cdc2}-like protein level is high in the meristem of seedling wheat leaf and declines, as cell differentiation proceeds, to a basal level in fully differentiated cells that is one sixteenth of that in meristematic cells (John et al., 1990). A particularly precise correlation was detected between the region in which p34^{cdc2} is catalytically activated and the region in which active cell division occurs (Fig. 5.1 and Fig. 5.3). Thus, although presence of p34^{cdc2}-like protein is a necessary precondition for division a crucial determinant of division is the mechanism by which p34^{cdc2} is converted into active holoenzyme. There is biochemical evidence that wheat p34^{cdc2} is complexed with 56 kDa cyclin like protein (John et al., 1993a) but the possible role of phosphorylation in controlling activity remains to be investigated.

The activity described here as p34^{cdc2}-like is that recovered by p13^{suc1} affinity chromatography. This fraction might in principle contain other CDKs that could have affinity for p13^{suc1} (Meyerson et al., 1992). There are several reasons for thinking that this possibility does not seriously call into question the conclusion that p34^{cdc2} activity is limiting for division. First activity is essentially zero in regions of the leaf where divisions do not occur, therefore even if Cdc2 activity contains a proportion of other CDKs the

decline is certain. Second, CDKs are in general less active than Cdc2 since it is at mitosis that phosphorylation is most abundant (Karsenti et al., 1987; Harper et al., 1990). Third CDKs appear to be far less abundant than Cdc2 judging by immuno-precipitation from human cells (Tsai et al., 1991) and in the present study of CDK mRNA was also found to be rare since no wheat CDKs were recovered by RT-PCR cloning, which yielded two *cdc2*-like fragments from 8 sequenced isolates (Chapter 3). It would be interesting to attempt more specific recovery of p34^{cdc2} using antibody against the carboxy-terminal region which has been effective with yeast and animal p34^{cdc2}, however this approach has been reported as unsuccessful with maize p34^{cdc2} (Colasanti et al., 1993) and may therefore also be unsuccessful with the closely related wheat enzyme.

The decline in p34^{cdc2}-like protein at the meristem margin is probably due to the cessation of accumulation of p34^{cdc2} which results in a dilution of p34^{cdc2} by other proteins. In Chapter 3, it was shown that high expression of *cdc2* mRNA is restricted to meristem cells, with *cdc2* mRNA undetectable in middle leaf segments (30-40 mm) where cells have stopped division and are undergoing differentiation. Similarly *cdc2* mRNA was not detectable in leaf tip segments (80-90 mm) where cells are fully differentiated. Thus cessation of Cdc2 protein accumulation may be largely under transcriptional control. Comparing the level of *cdc2* mRNA to the level of p34^{cdc2} protein, the biggest disparity was in the region 30-40 mm above the leaf base, where *cdc2* mRNA was undetectable (Fig. 3.13) but p34^{cdc2}-like protein was relatively abundant relative to total soluble proteins being at 30% of the level found in meristem cells (Fig. 5.4). The simplest explanation is that absence of *cdc2* mRNA had enforced cessation of *cdc2* protein synthesis but the protein maybe relatively stable in this tissue and had not yet become fully diluted by accumulation of other proteins.

Persistence of a basal level of *cdc2*-like protein in differentiated cells, at about 6% of the maximum level, which is seen in active meristem cells (Gorst et al., 1991), is of uncertain significance. Residual synthesis of the protein could persist if *cdc2* mRNA is present at very-low, rather than absolutely zero levels. Furthermore a protein can be greatly reduced in relative level by accumulation of other proteins but not completely

removed. There is additionally a possibility that wheat cells contain other PSTAIR-containing enzymes that could contribute to the signal from PSTAIR antibody. This possibility may be considered unlikely from evidence in Chapter 3 that PCR primers capable of amplifying variants of *cdc2* were only detected to have amplified *cdc2* but not *cdk2*-like genes. Also a biological significance of retaining a Cdk2-like enzyme, which is the predominant alternative PSTAIR-containing enzyme in animal cells, is unlikely since cells could not proliferate without Cdc2 but could only enter S phase and it is known that wheat leaf cells arrest in G1 phase (Wernicke and Milkovits, 1987). However this possibility is not eliminated.

A novel feature of the present study is that *cdc2* mRNA and *cdc2* enzyme level were compared in plant tissue. Others have measured only *cdc2* mRNA in meristematic and non-meristematic regions of maize leaf (Colasanti et al., 1991), and radish and *Arabidopsis* root (Martinez et al., 1992; Hemerly et al., 1993), and different organs of alfalfa (Hirt et al., 1993). A correlation of raised *cdc2* mRNA level with division has also been noted during the stimulation of proliferation in dicotyledonous tissue, such as soybean (Miao et al., 1993) and pea root (John et al., 1993b). In view of this evidence it has been presumed that *cdc2* transcription in plants correlates with potential for cell division (Heremerly et al., 1993) however this makes the assumption that the mRNA would be translated without extensive post transcriptional control. The present results confirm that indeed in the seedling wheat leaf the region of highest mRNA levels is the region of highest concentration of p34^{cdc2}-like protein relative to other proteins. As noted above however the protein can persist at significant levels in cells that are not actively dividing but have begun to differentiate. The changes of *cdc2* expression stage and p34^{cdc2} level in the transitional region from meristem to differentiating cells in wheat seedling leaf indicate that cessation of *cdc2* gene expression causing low level of p34^{cdc2} protein may have a role in the plant development, by enforcing, in cells leaving meristem, a switch from proliferation to the differentiation of mature photosynthetic cells.

The decline of p34^{cdc2} protein in mature wheat cells is particularly significant since it becomes irreversible. The culture of wheat seedling segments showed that only meristematic cells or cells just adjacent to the meristem region of the leaf were able to

respond to the presence of the auxin analogue 2,4-D by dividing and forming callus (Fig. 5.7). When transferred to medium with low 2,4-D concentration (0.5 μM) only cells containing active p34^{cdc2} and high level of p34^{cdc2} protein could be stimulated to continue division, all other tissue was unresponsive. At higher concentrations of 2,4-D (50-150 μM) cells immediately adjacent to the meristem (8-12 mm) that had recently ceased dividing and containing little active p34^{cdc2} but significant levels of the inactive enzyme protein could be stimulated to activate the protein (compare Fig. 5.11 a and d) and resume division (Fig. 5.7, Segment 5). However auxin did not induce any increase in level of p34^{cdc2}, it only stimulated partial retention of p34^{cdc2} (Fig. 5.9). Clearly conditions for inducing p34^{cdc2} synthesis are more exacting than those required for activation of existing enzyme and were not well provided in culture.

This is in contrast with observations in dicotyledonous plants. Auxin-stimulated de-differentiation in excised tissue from mature cotyledons of the dicotyledonous carrot, was accompanied by increase in level of p34^{cdc2}-like protein restoring the level to that of dividing cells, and the responding cells re-entered the cell cycle and consequently formed a callus (Gorst et al., 1991). It was also found that the phytohormone induced elevation of p34^{cdc2}-like protein level in tobacco pith culture (John et al., 1993b; Zhang et al., 1996). The mechanism of these increases is probably increased rate of translation since a rapid elevation of *cdc2* gene expression has been found in response to environmental wounding, light or phytohormone stimulation, in *Arabidopsis*, alfalfa, soybean and pea (Hemerly et al., 1993; Magyar et al., 1993; Miao et al., 1993; John et al., 1993b). These responses of tissues from dicotyledonous plants are in contrast with the inability of mature wheat cells to resume division when cultured on nutrient medium supplemented with auxin. The difference of *cdc2* gene response to auxin treatment between dicotyledonous plants and monocotyledonous plants may explain at the molecular level the inability of monocotyledonous plants to readily resume cell division.

A change in p34^{cdc2} protein level has been found to correlate with active division or quiescence in animal cells (Lee et al., 1988), in plants (John et al., 1990; Gorst et al., 1991) and it has been proposed that change in *cdc2* expression participates in control of cell development (Lee et al., 1988; Krek and Nigg, 1989; John et al., 1993a, b; Hemerly

et al., 1993). This relatively slow acting form of control could be important during plant growth, in which the switch from cell division to differentiation is regulated during organ formation and during the maturation of cells newly formed by a meristem. A faster acting control, or fine control, over p34^{cdc2} activity during progress through the cell cycle appears to be imposed by other *cdc* proteins, such as cyclins, which can be subject to rapid turnover and are essential components of the active p34^{cdc2} holoenzyme complex. Ferreira et al.(1994 a, b) found that expression of a cyclin gene (*cyc1At*) in *Arabidopsis* is almost exclusively confined to dividing cells and a similar finding is reported by Fobert et al. (1994) in inflorescence apices of snapdragon. It has therefore been suggested that cyclin levels control division in plant cells (Ferreira et al., 1994 a, b) although in yeast and somatic animal cells enzymes that control p34^{cdc2} phosphorylation are usually rate limiting (Nurse 1990). The present data for p34^{cdc2} kinase in dividing cells of wheat leaf meristem are consistent with the location of the protein being a determinant of capacity for proliferation and additionally with a fine control of catalytic activity being able to restrict the occurrence of division in cells that contain the Cdc2 protein. p34^{cdc2} protein level dropped as cells ceased division and underwent differentiation in wheat seedling leaf, but it never completely disappeared. In contrast, p34^{cdc2} kinase activity was almost exclusively confined to dividing cells and dropped to zero at the margin of the meristem which, it may indicate that negative regulatory factors operating a fine control of p34^{cdc2} kinase activity are important to plant development. The molecular basis of this fine control requires further investigation in plants. It is possible but as yet unproven that plants contain homologues of Wee1 or CKIs.

To test the proposition that the decline in level of *cdc2*-like enzyme relative to other proteins is significant in the control of plant cell division, a converse developmental situation was sought, in which cell proliferation is initiated as part of developmental program. The initiation of root growth during pea germination was selected.

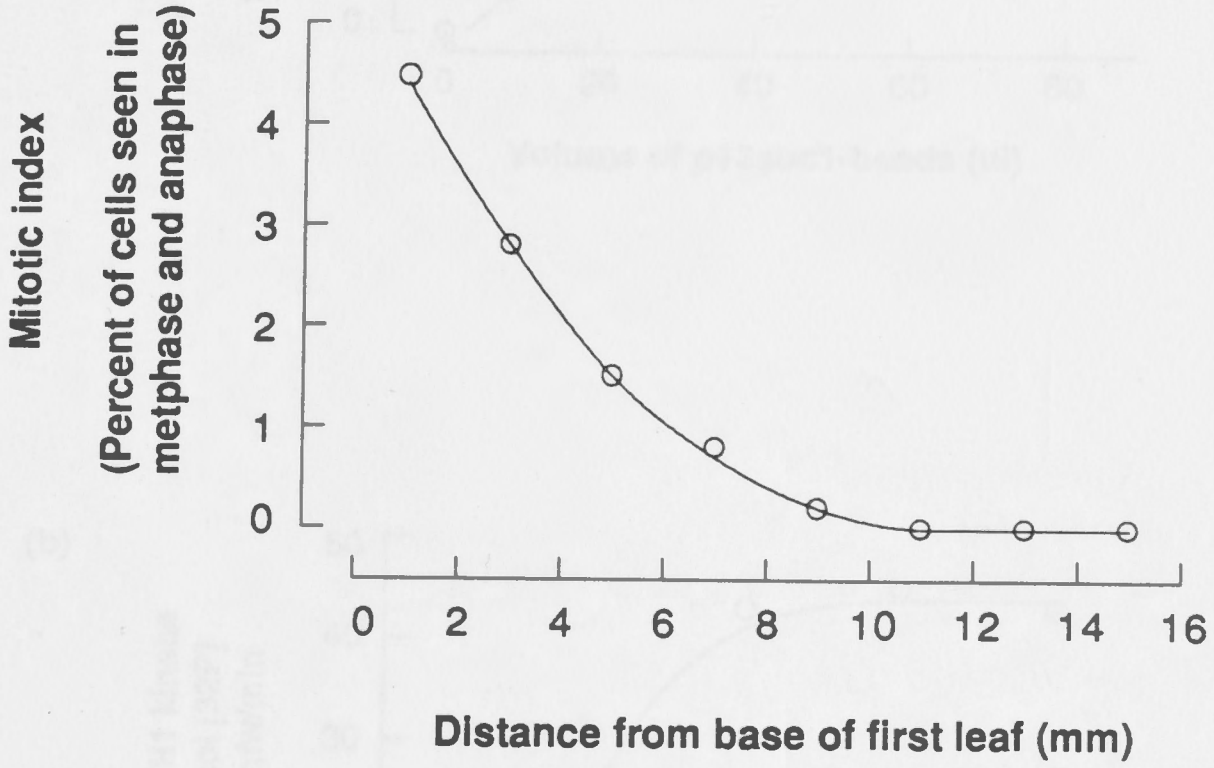
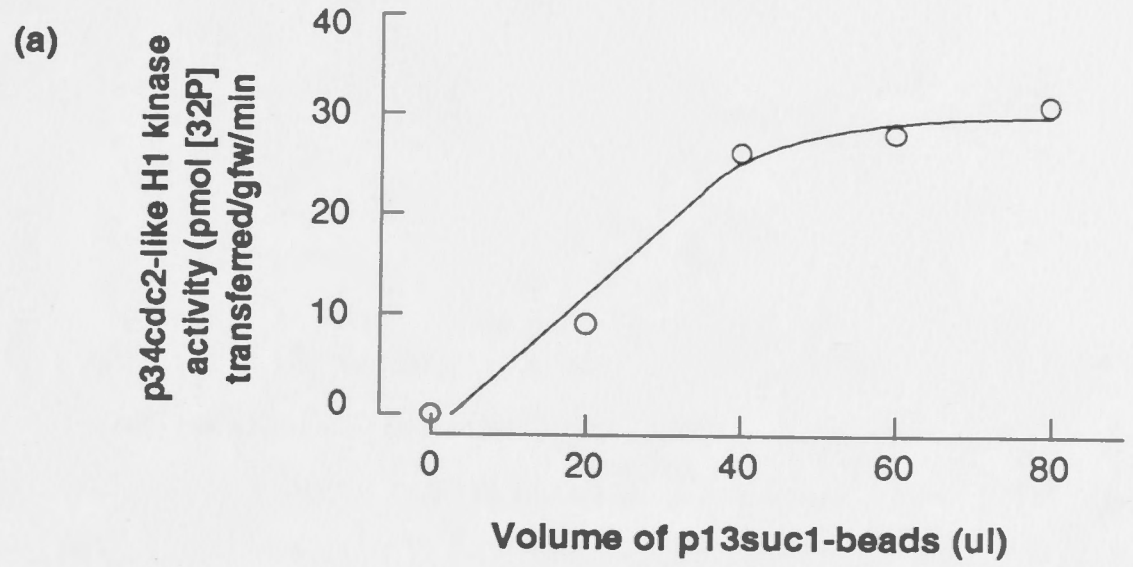


Fig. 5.1 Mitotic index of 7-day-old wheat first leaf in the basal region. Segments of 2-mm long were taken contiguously from the leaf base, fixed in 3:1 ethanol :acetic acid solution and stained in acetocarmine (Method in 2.2.2). A minimum of 2000 cells was evaluated in each sample.



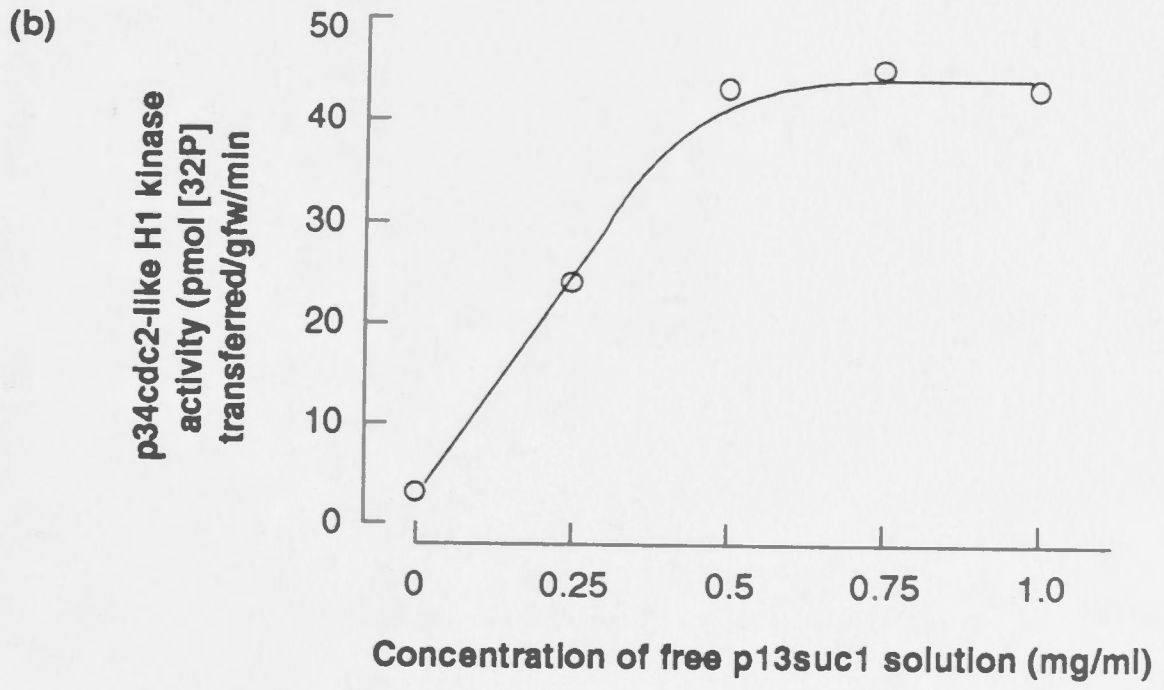
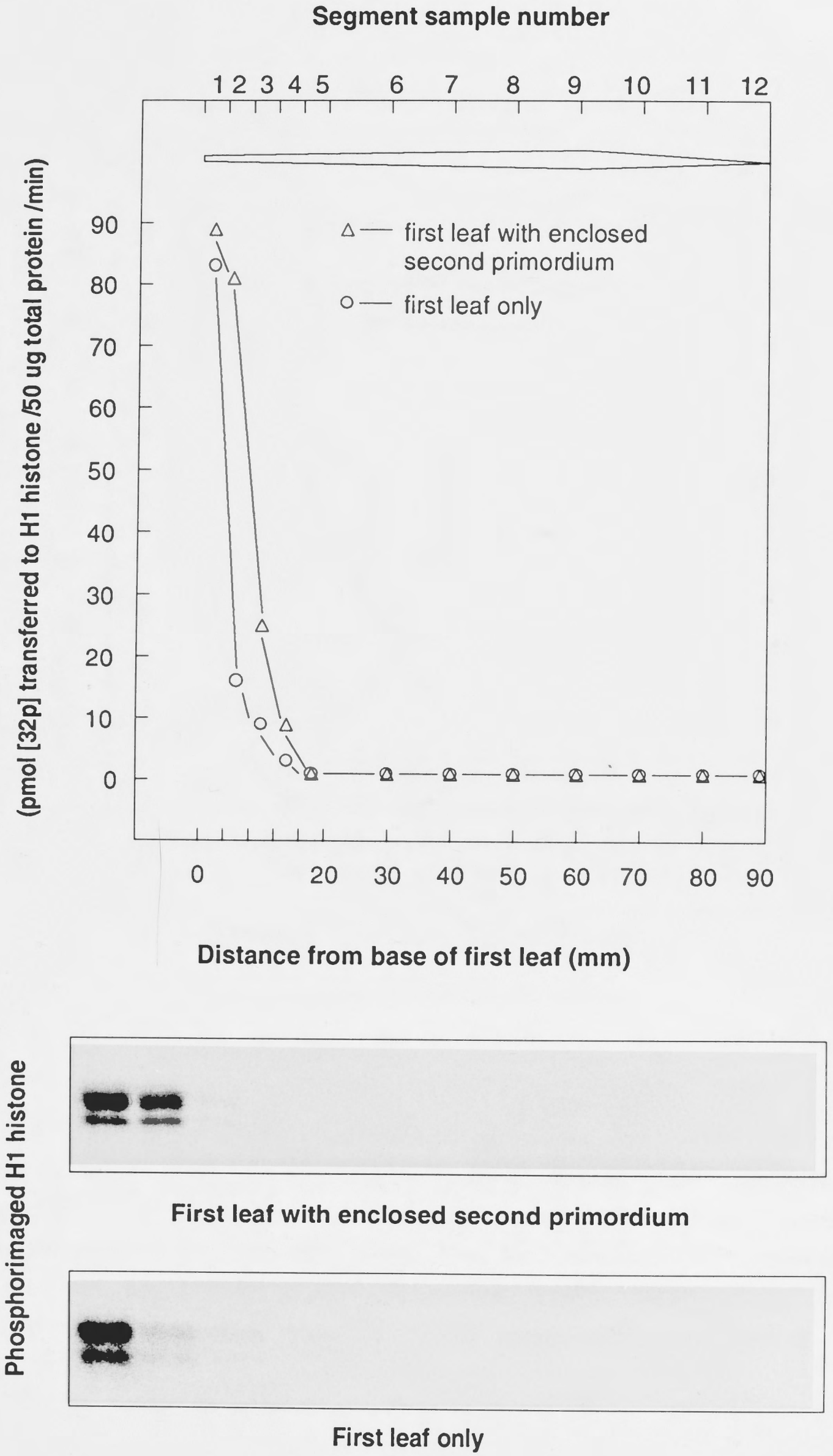


Fig. 5.2 Recovery of p34^{cdc2}-like enzyme by increasing volume of p13^{suc1}-beads (a), and elution of p34^{cdc2}-like enzyme by increasing concentration of free p13^{suc1} solution (b) from extract containing 1.2 mg wheat meristem protein (from 0.05 g fresh weight).

(a) p34^{cdc2}-like H1 kinase activity recovered by different volumes of p13^{suc1}-beads, using 0.5 mg/ml free p13^{suc1} for elution prior to assay.

(b) p34^{cdc2}-like H1 kinase activity recovered by 40 µl p13^{suc1}-beads and different concentrations of free p13^{suc1} for elution prior to assay.

Fig. 5.3 p34^{cdc2} kinase activity in 4 mm segments of first seedling leaf taken contiguously between 0-20 mm from the leaf base and above this at 10 mm intervals (see John et al., 1990). H1 histone kinase activity was measured after affinity purification of the enzyme using p13^{suc1} (Brizuela et al., 1987) and the labelled histone was electrophoretically separated and quantified as described in Chapter 2 (2.2.5).


Segment sample number
1 2 3 4 5 6 7 8 9 10 11 12
p34cdc2-like kinase activity
(pmol [32p] transferred to H1 histone /50 ug total protein /min)
90
80
70
60
50
40
30
20
10
0
first leaf with enclosed second primordium
first leaf only
0 20 30 40 50 60 70 80 90
Distance from base of first leaf (mm)
Phosphorimaged H1 histone
First leaf with enclosed second primordium
First leaf only

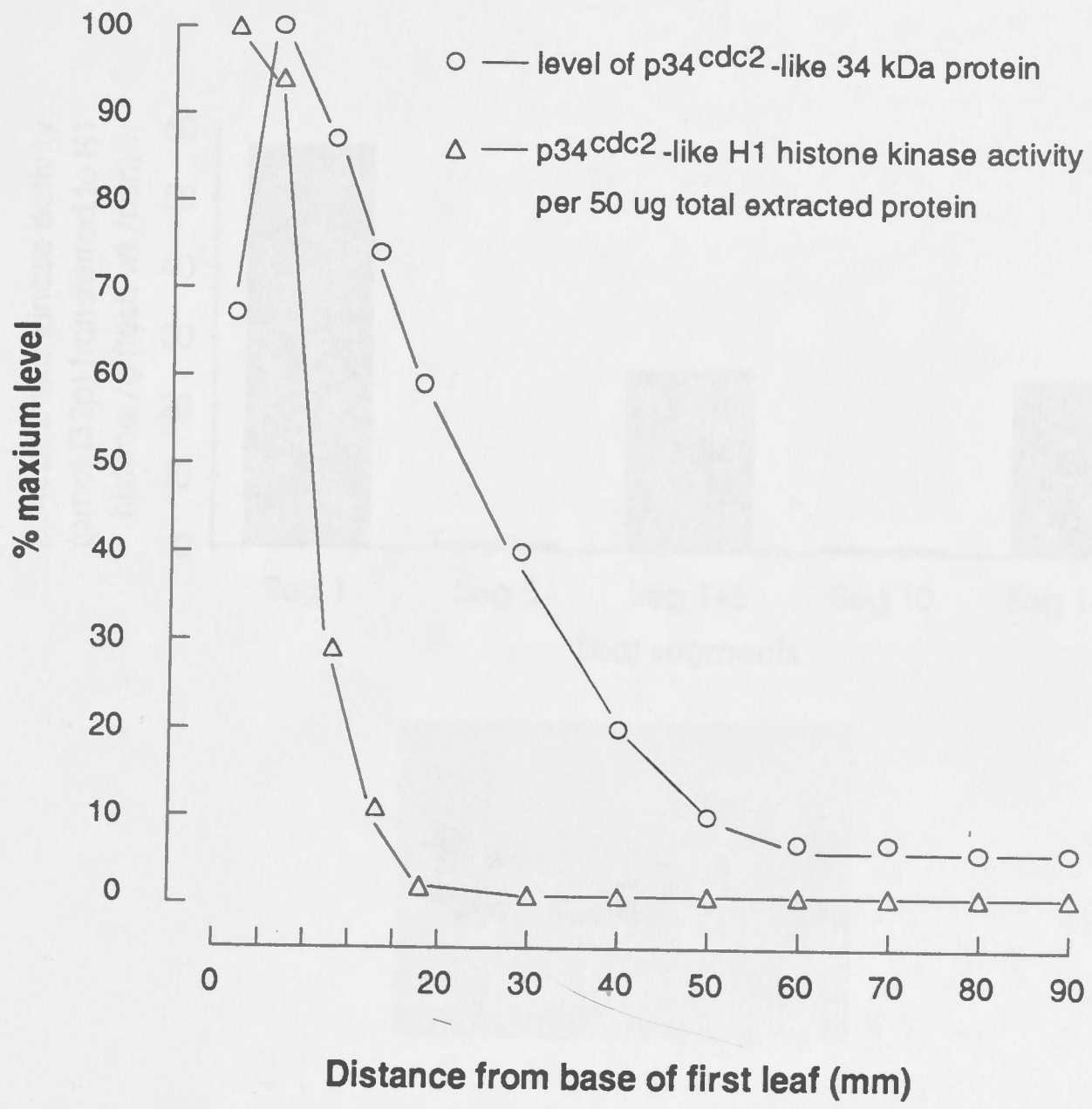


Fig. 5.4 The relative levels of p34^{cdc2}-like protein (o) and p34^{cdc2}-like kinase activity (Δ) in the developing first leaf of 7-day-old seedling wheat. The data for amount of enzyme protein are derived from John et al. (1990) and have also been confirmed in the present study (Fig. 5.9a). Data for kinase activity are taken from Fig. 5.3. Both measurements of p34^{cdc2}-like protein level and kinase activity are for first leaf with enclosed second primordium and therefore are directly comparable.

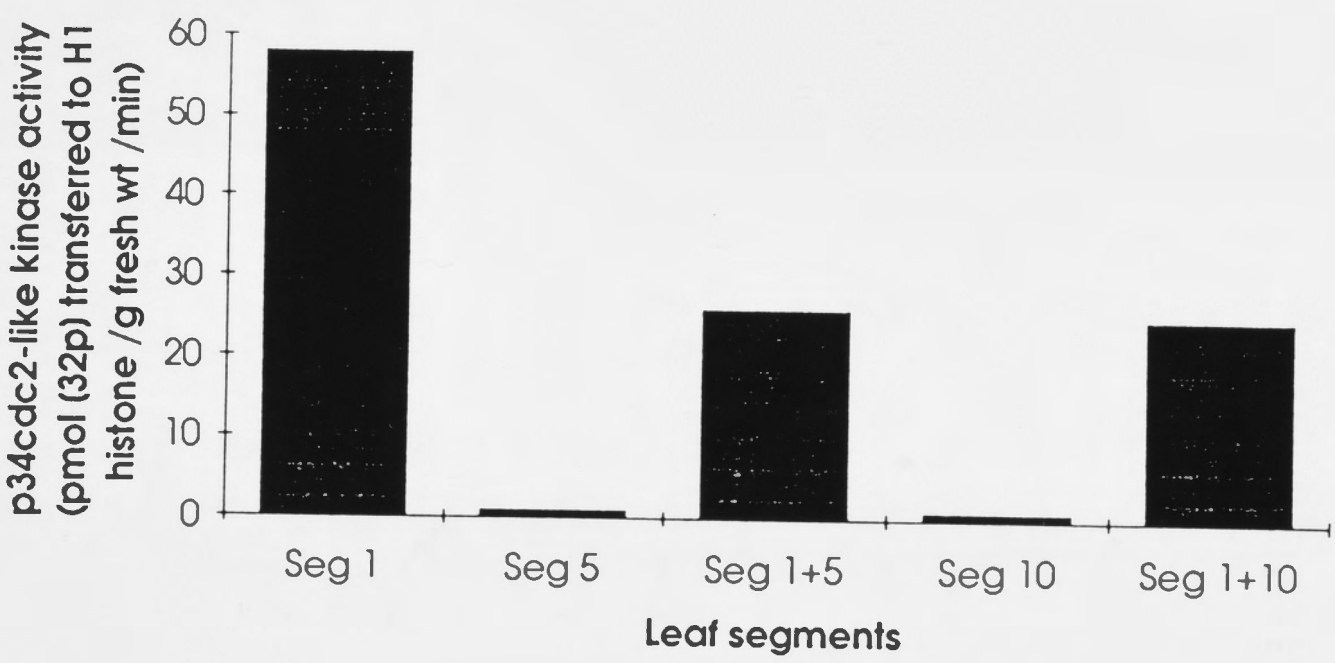
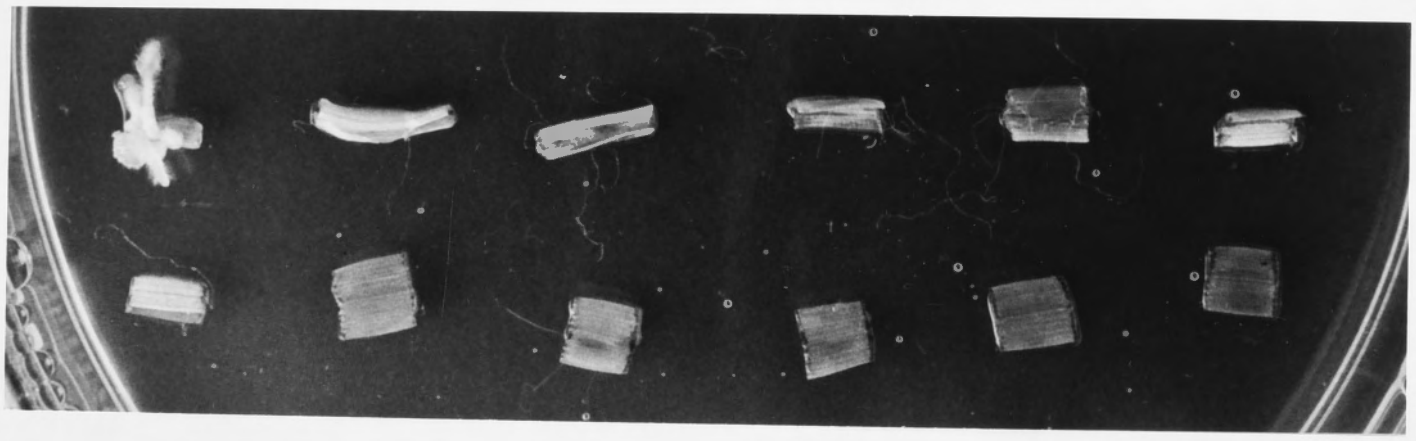
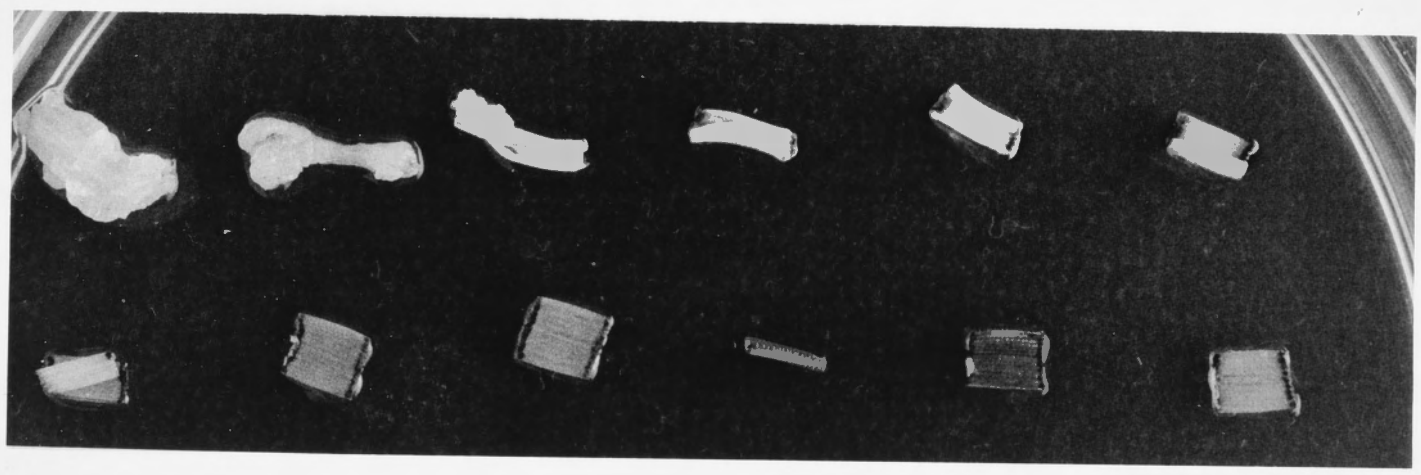


Fig. 5.5 p34^{cdc2} kinase activity in extracts from segment 1, where cells were dividing actively (see Fig. 5.1), and from segments 5 and 10 which were at 20 mm and 70 mm from the base of the leaf in regions where cells had ceased dividing recently or 2 days earlier. Also shown are activities found in extracts from equal weight mixtures of liquid nitrogen-ground cells from segments 1 and 5 (Seg 1+5) and from segments 1 and 10 (Seg 1+10).



0.6 μM 2,4-D



40 μM 2,4-D

Fig. 5.6 *In vitro* culture of 4 mm long segments cut from 7-day-old wheat seedling leaf as shown in Fig. 5.3. Segments were excised under sterile conditions and cultured on MS agar medium with concentrations of 2,4-D shown. In each picture segment 1 is placed at the left of the upper row with the others in numerical order. At low concentration of 2,4-D (0.6 μM) the first segment formed callus and adventitious roots and the second and third segments elongated. At 40 μM 2,4-D the first three segments formed callus but not roots. Photograph taken after culture for 2 weeks.

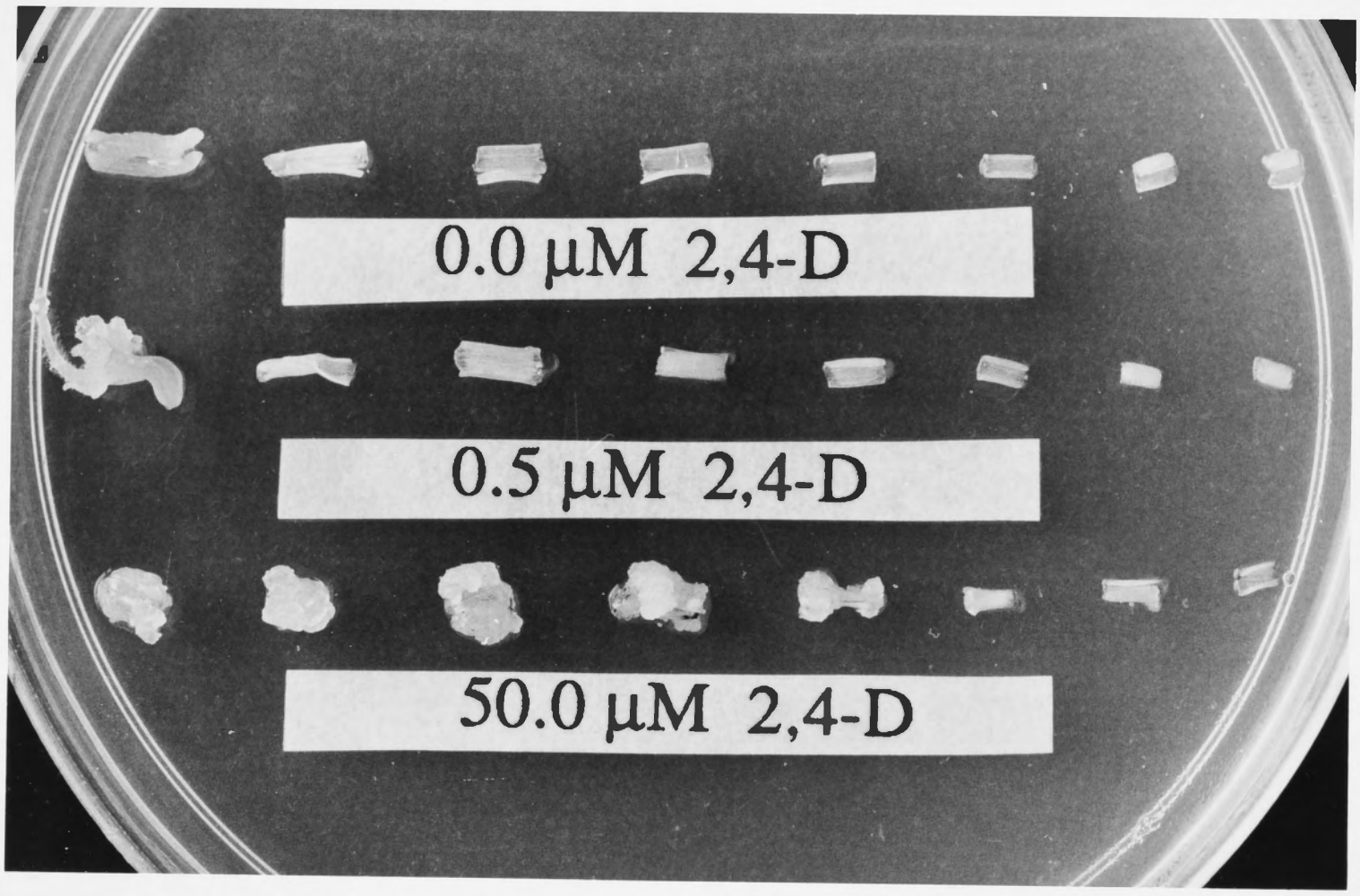


Fig. 5.7 *In vitro* culture of 2 mm long segments cut from the base of the first leaf of seedling wheat. Segments were taken under sterile conditions from 7-day-old first leaf base 0-16 mm then cultured on MS agar medium with the concentrations of 2,4-D shown and have been moved into a single dish for photography. In each row segment 1 is placed at the left with the others in numerical order. The first five segments (from the 0-10 mm region) could respond to auxin by forming callus on the medium with 50 µM 2,4-D (bottom row), however the callus from all segments remained disorganised at this hormone concentration and no formation of roots was seen. On the media with lower concentrations of 2,4-D (0.5 and 2.5 µM), segment 1 could form callus with adventitious roots (2.5 µM not shown; 0.5 µM shown in middle row). Tissue was photographed after culture for 3 weeks.

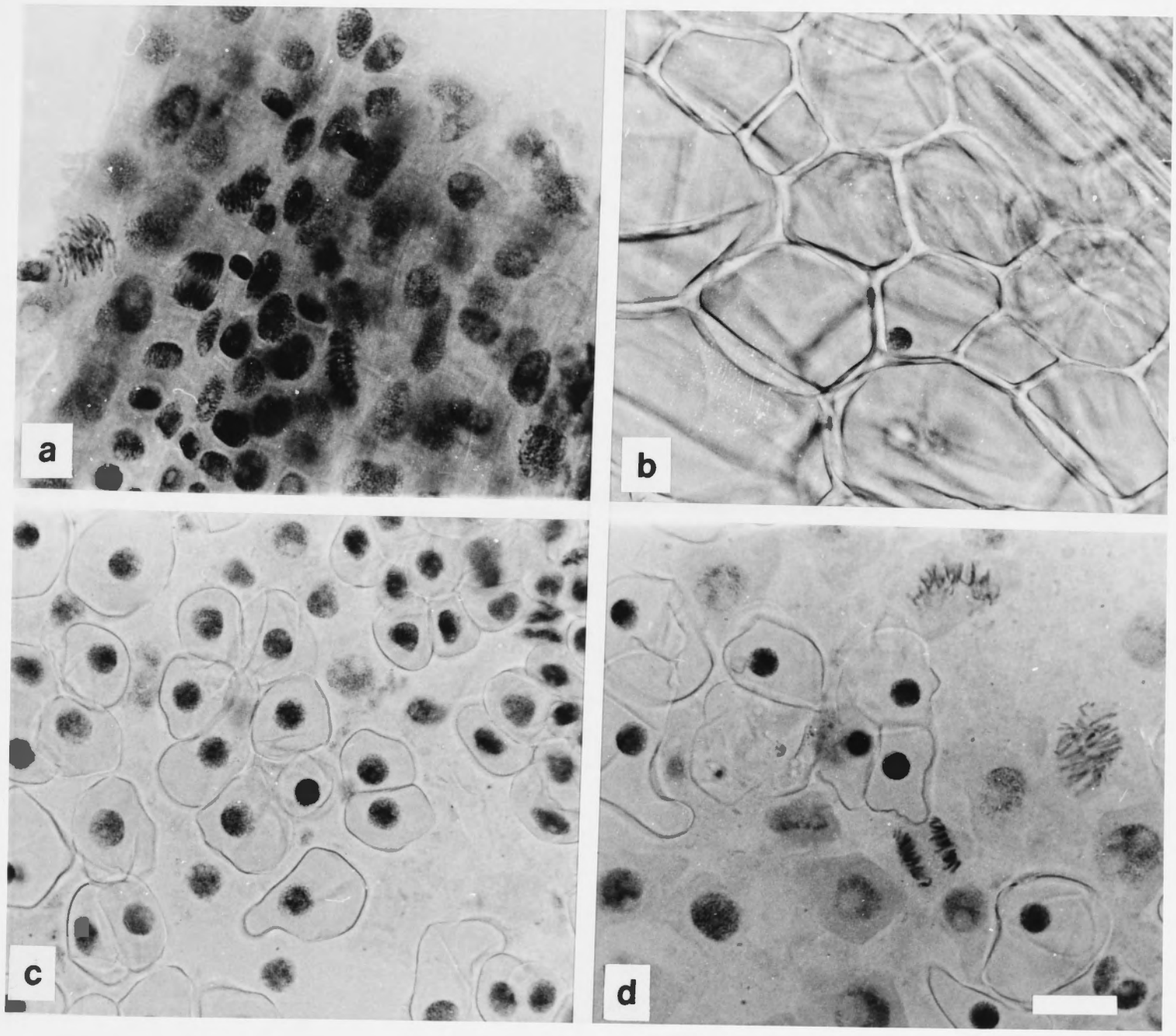


Fig. 5.8 Alignment, structure and division activity of meristem cells of the leaf and callus cells formed *in vitro* culture on MS medium with 50 μM 2,4-D. Seedling wheat leaf meristem tissue or callus formed from this tissue was fixed and stained with acetocarmine. The stained tissues were squashed onto microscope slides and photographed.

(a) Meristem cells from the intact leaf were regularly arranged, rectangular in shape with dense nuclei. Cells in various mitotic phases can be seen.

(b) Meristem tissue after culture on MS medium without phytohormone for 3 weeks. Cells were expanded, vacuolated, and no mitotic activity was discernible.

(c) and (d) Meristem tissue cultured on MS medium with 50 μM 2,4-D for 3 weeks. Cells lost regular relative placement and became unorganised callus, in which cells were round with dense nuclei, some cells were in mitotic phases (d). Callus cells were bigger than meristem cells and their mitotic activity (about 1% in mitosis) was lower than that of intact leaf meristem (4.6% in Fig. 5.1).

All pictures are at the same magnification. Scale bar, 10 μm.

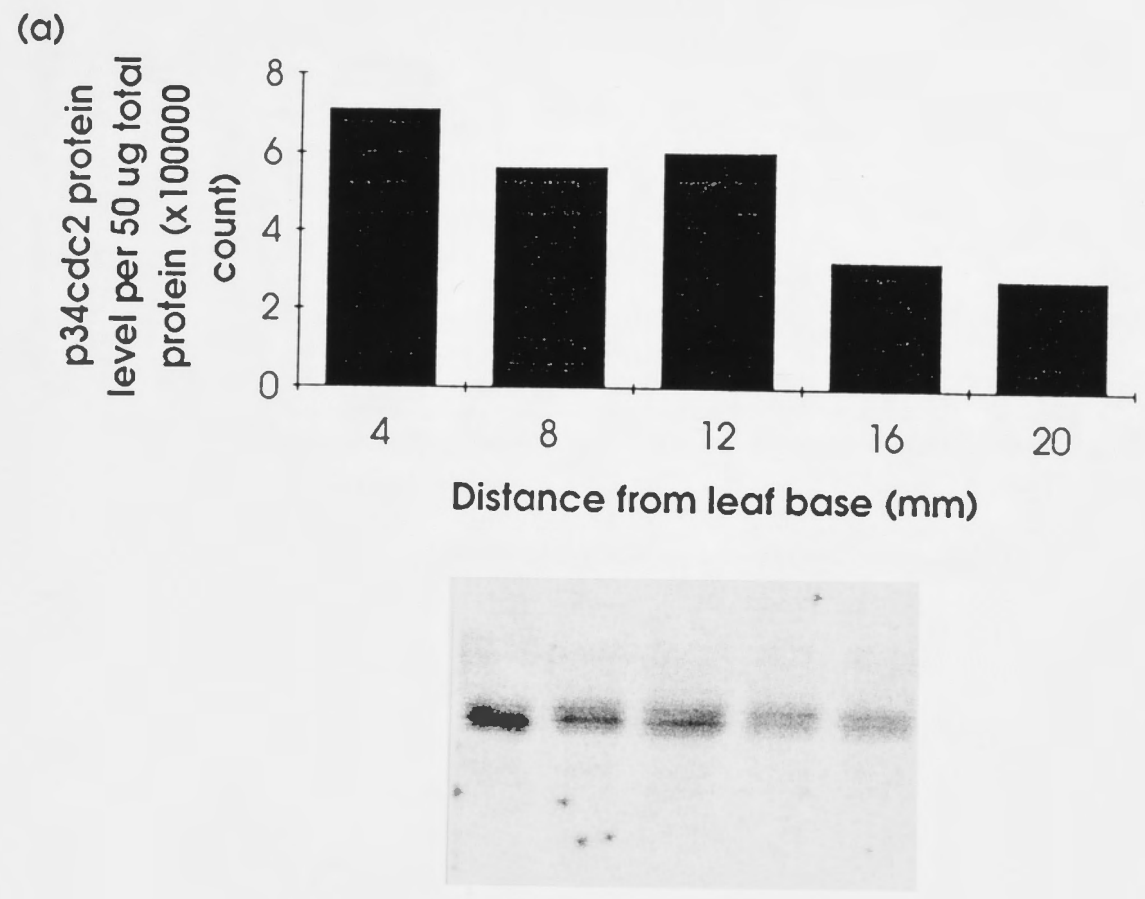
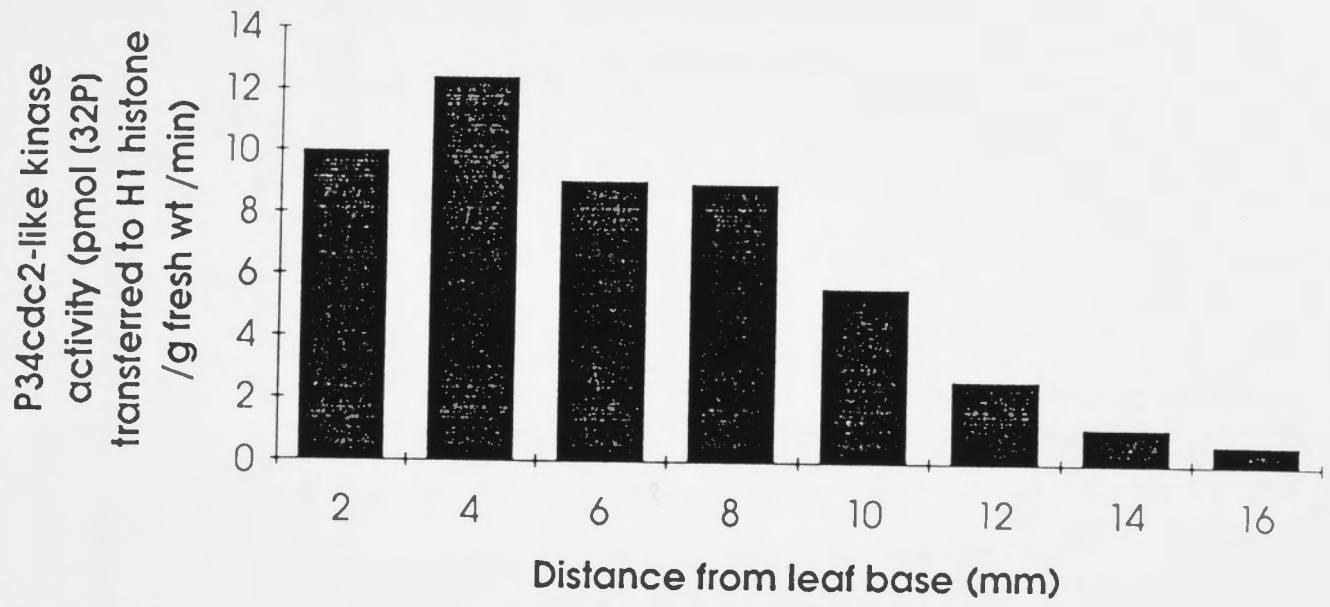


Fig. 5.9 p34^{cdc2}-like protein level in seedling wheat leaf before culture (a) and after 3 weeks culture (b). Protein of 34 kDa was estimated on Western blots with PSTAIR antibody.

(a) p34^{cdc2}-like protein level in 4 mm long segments from 0-20 mm above the leaf base before culture.

(b) p34^{cdc2}-like protein level in callus formed by 2 mm segments cut from the leaf base 0-16 mm after culture on MS medium with 50 μM 2,4-D for 3 weeks.

(A)



(b)

(c)

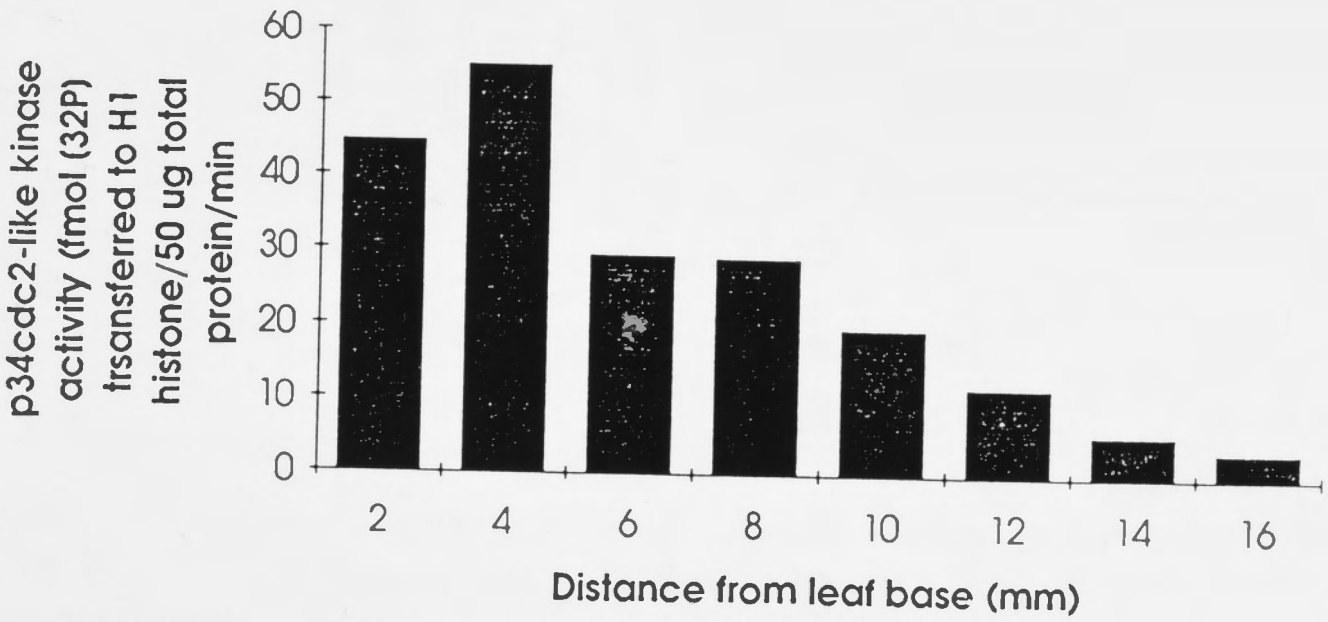


Fig. 5.10 p34*cdc2*-like kinase activity in callus formed by 2 mm segments cut from the leaf base region (as in Fig. 5.9 b) after culture on MS medium with 50 µM 2,4-D for 3 weeks expressed in (a) and (b) as activity per g. f. w. and in (c) as activity per 50 µg extracted total protein.

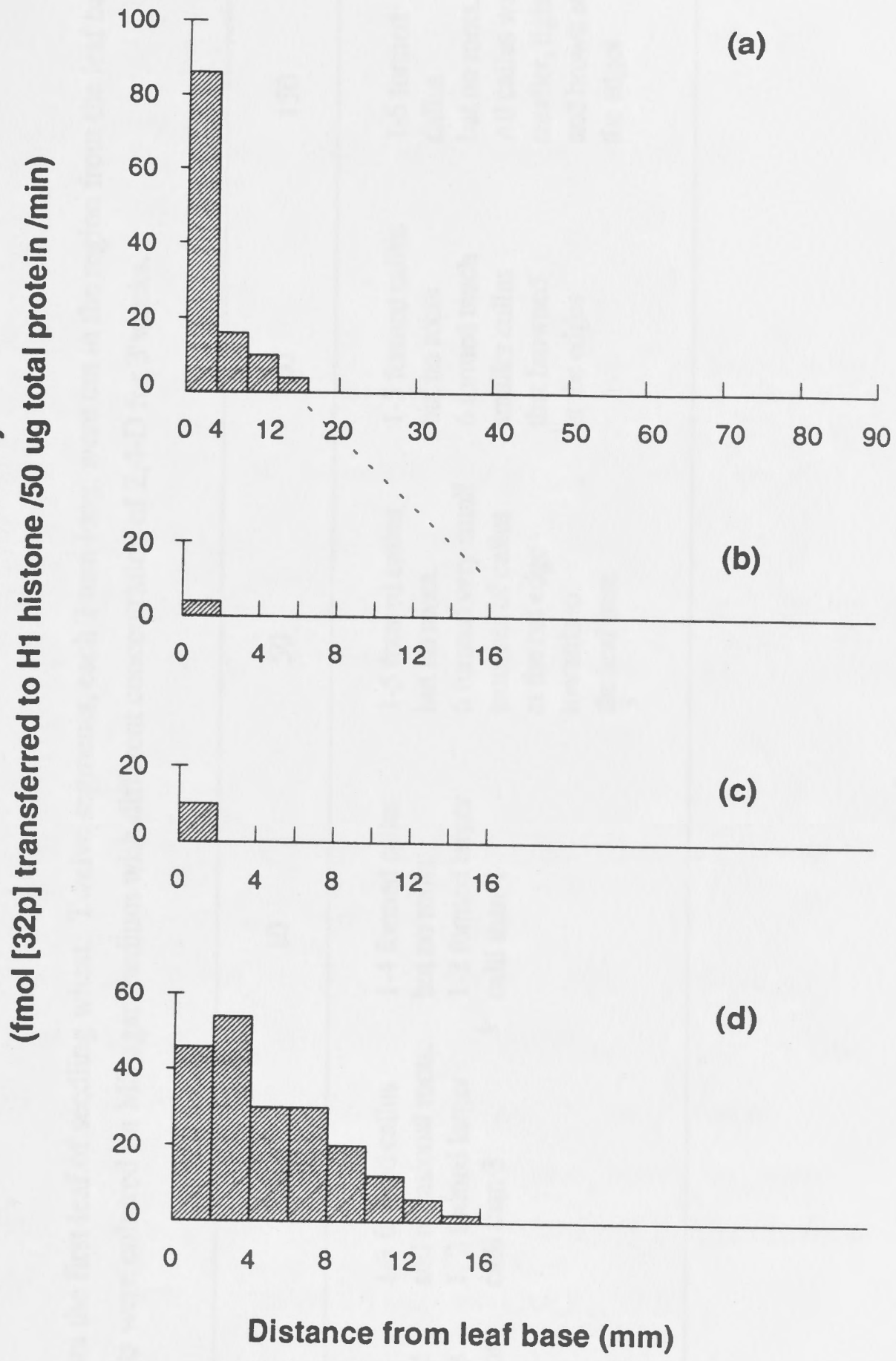


Fig. 5.11 A comparison of p34^{cdc2}-like kinase activity in the p13^{suc1}-purified fraction from seedling wheat leaf with that in the same region after excision and culture for 3 weeks.

(a) 7-day-old first leaf segments as removed from the leaf and before culture.

(b) 2 mm long segments from the 0-16 mm region of leaf base after culture on MS medium without auxin (zero 2,4-D).

(c) 2 mm long segments as in (b) cultured on MS medium with 0.5 μM 2,4-D.

(d) 2 mm long segments as in (b) cultured on MS medium with 50 μM 2,4-D.

Note that the low activity seen in (c) was sufficient to support callus and root formation shown in Fig. 5.7.

Table 5.1 Culture of tissue excised from the first leaf of seedling wheat. Twelve segments, each 2 mm long, were cut in the region from the leaf base to 24 mm above the base. The segments were cultured on MS agar medium with different concentration of 2,4-D for 3 weeks.

2,4-D (μM)	0	0.5	2.5	10	50	100	150
segment response to 2,4-D (segments numbered as in Fig. 5.3)	1-6 elongated	1-6 enlongated 1 formed a small callus and roots	1-3 formed callus and occasional roots. 1, 2 formed larger calli than 3	1-4 formed callus but no roots. 1-3 formed larger calli than 4	1-5 formed callus but no roots. 6 formed very small amounts of callus at the cut edge towards to the leaf base	1-5 formed callus but no roots. 6 formed much smaller cullus that browned at the edges	1-5 formed callus but no roots. All callus was smaller, tighter and brown at the edges

Chapter 6

Cdc2 Involvement in Pea Root Development

6.1 Introduction

Chapter 5 describes a close correlation of level and activity of the *cdc2* gene product p34^{cdc2} kinase, in seedling wheat leaf, with both the occurrence of cell division and the capacity for continued cell proliferation in tissue culture. To test this correlation further another experimental system was chosen to allow investigation of changes associated with the initiation of cell proliferation. The developing pea root provided this opportunity as well as a further example of the cessation of division in the cell elongation region behind the root tip, which could parallel changes seen adjacent to the leaf meristem if common mechanisms are involved.

Roots are fairly simple structures containing relatively few cell types. Stages of cell development are seen in a continuum along the length of the root, from tip meristem basipetally to fully differentiated tissue. The garden pea (*pisum sativum*) has been used extensively in previous studies of cell development in roots (Brown and Broadbent, 1950; Chaly and Setterfield, 1975), and also in relation to phytohormone transport in roots and the relationship between hormones and lateral root formation (Torrey 1956; MacLeod 1973; Wightman and Thimann, 1980; Wightman et al., 1980). In this Chapter, I investigated the distribution of p34^{cdc2} protein and its kinase activity during the course of pea root development. A valuable comparison with wheat leaf was provided by the opportunity in pea to study the initiation of cell division.

6.2 Materials and Methods

Experiments were conducted with seedlings of garden pea, *Pisum sativum* L. cv. Alaska. The seeds were surface sterilised by treatment with 10% sodium hypochlorite for 20 min, followed by 5 rinses with large volumes of sterile distilled water. The seeds were allowed to imbibe in aerated sterile water for 8 h then planted in 10 cm deep wet vermiculite in a tray. To ensure straight primary roots, care was taken that each seedling was planted with the radicle pointing downwards. The seeds were covered with 1 cm of moist vermiculite and the tray covered with aluminium foil. The seeds were then incubated at 25 °C in darkness.

To analyse the time course of root development, radicles/roots were taken at 16, 24, 32, 44, 56, 68 h after imbibition. The seedlings were carefully removed from the vermiculite and rinsed in distilled water, and then blotted briefly on a filter paper. The whole radicle/root was cut off by razor blade and immediately frozen in liquid nitrogen. The range of lengths that included 95% of the population is indicated in Table 6.1a and unusual roots outside these ranges were discarded.

To analyse the distribution of p34^{cdc2}-like protein and enzyme activity within developing roots, roots of 3-day-old pea seedling that were about 40 mm long were cut transversely into 2-mm segments in the first 10 mm from the tip and further from the tip into 5-mm segments (see Table 6.1 b and Fig 6.3 a). The segments were frozen in liquid nitrogen, then ground in liquid nitrogen and the total protein was extracted by adding 300 µl NDE buffer (see 2.2.5.2) to 0.1 g grindate and vortexing then centrifuging for 5 min at full speed in a microfuge at 4 °C before retaining the supernatant. Of this, 130 µl was routinely taken for affinity purification of p34^{cdc2}-like proteins for p34^{cdc2} kinase activity assay as described in Chapter 2 (2.2.5.2); 20 µl of extract was assayed to determine the concentration of total protein in the extract (methods see 2.2.3.2); an appropriate volume of each extract which contained about 50 µg total protein was used for Western blot determination of 34 kDa PSTAIR protein as described in Chapter 2 (2.2.3.3 and 2.2.3.4). Each experiment was repeated.

6.3 Results

6.3.1 p34^{cdc2}-like protein level and p34^{cdc2}-like H1 histone kinase activity in the developing pea root

The length and protein content of the emerging radicle during germination is shown in Table 6.1. The growth rate (elongation rate) of roots was faster after 56 h, consistent with the finding of Wightman (see Fig 6.5) that the total cell number in the root did not increase during the first 48 h (Fig 6.5), while elongation of the root was due to the cell elongation. Table 6.1 (a) also shows that the protein concentration was highest at 16 h sample and then decreased as cells elongated and became more vacuolate.

p34^{cdc2}-like protein level during the course of root development is shown in Fig. 6.1. As a proportion of total protein the amount of p34^{cdc2}-like protein increased linearly between 16 h and 68 h after imbibition. This is consistent with synthesis of p34^{cdc2} protein being an early event leading to cell division in the root meristem. The p34^{cdc2}-like kinase activity during the same time period reached a maximum level by 56 h after imbibition (Fig. 6.2) which was about twice the activity seen at 32 h and 44 h and about 10 times the 16 h activity. Attainment of full activity of p34^{cdc2}-like kinase coincided with the establishment of the full rate of cell number increase which was attained at about 60 h.

The level and activity of p34^{cdc2}-like protein kinase during root development were all based on 50 μg amounts of extracted total protein. As a comparison, the level of p34^{cdc2}-like protein in the 56-h sample was 3 times the 16-h level, whereas the kinase activity in the 56-h sample was 10 times the 16-h level. Thus an early phase of p34^{cdc2} accumulation is followed by one of activation in which enzyme molecules formed earlier in development are activated as cells move to a phase of active cell cycle progress.

6.3.2 p34^{cdc2}-like protein level and p34^{cdc2}-like H1 histone kinase activity distribution in the root

Three-day-old pea roots were cut (as shown in Fig. 6.3a) into 11 segments 2-mm long in the first 10 mm from the tip and segments 5-mm long in the remainder. The total protein concentrations of these segment extracts are listed in Table 6.1 (b) and show that the cytoplasm of cells in the embryonic tip was highly concentrated. The p34^{cdc2}-like protein level measured in these segments relative to other proteins is a little higher at the tip than in other segments (Fig. 6.3). A slight reduction in p34^{cdc2} level in segments 2-5 (2-10 mm from the tip) correlates with the transition to the elongation zone in which cell enlargement rather than division occurs. A striking difference was seen in the profile of activity of this enzyme. Activity was considerably higher in the tip 0-2 mm than in other regions being three times higher than in the 4 mm to 40 mm region comparing equal amounts of extracted total root protein (Fig. 6.4 a), and eleven times higher comparing activity from equal fresh weight of tissue (Fig. 6.4 b).

6.4 Discussion

The developing pea root provides a valuable comparison with the wheat leaf in two ways. First it allows a positive test of the conclusion, drawn from negative evidence in the wheat leaf, that raised levels of p34^{cdc2}-like protein are important for cell division in plants. In the wheat leaf a decline of p34^{cdc2}-like protein relative to others is associated with a decline in capacity for cell division. The pea root allows a test of whether the initiation of cell division requires an increase in level of p34^{cdc2}-like protein relative to others. The second advantage of the pea root is that it contains a temporal gradation of cells progressing from active division at the apical meristem through elongation to differentiation. There is a parallel with the progress of cells leaving the leaf meristem (Chapter 5) and it is of interest whether a decline in level of p34^{cdc2}-like protein and activity is involved in the root as in the leaf.

The initiation of cell division in the radicle at germination is preceded by a period in which cells, formed during development of the seed, enter a process of elongation and differentiation with the result that there is a decline in the number of cells remaining isodiametric and with dense cytoplasmic contents. This decline is reflected in a decline of protein concentration per fresh weight (Table 6.1 a) and is most rapid during the first 48 h after imbibition. It leaves a core of about 100,000 small dense cells at the tip of the root which then enter division and function as an apical meristem that thereafter sustains a flow of cells through the elongation and differentiation program. The early phase, entirely of cell elongation, is reflected in a fourfold increase in radicle length within the first 48 h prior to any detected increase in cell number. The establishment of an active meristem is indicated by the initiation of cell number increase between 48 h and 60 h. Cell number continues to increase approximately linearly after 60 h (Fig. 6.5).

A positive correlation of increased accumulation of p34^{cdc2}-like protein with the initiation of division is indicated by the three fold increase of the protein relative to total extractable root proteins in the whole root during the first 56 h of germination (Fig. 6.1). The increase may well be greater in the region of central cells that becomes active as a meristem. Even averaged over all cells the data complement those in the preceding Chapter by underlining the capacity of plant tissue to regulate level of p34^{cdc2}-like protein and raise it when division becomes appropriate.

An even more precise correlation was observed between initiation of division and catalytic activation of p34^{cdc2}-like protein kinase. Activation accelerated from 24 h after germination and reached a plateau at 56 h (Fig. 6.2) when the full rate of cell number increase was fully established (Fig. 6.5). These observations of activity underline the conclusion drawn from the wheat leaf that a fine control of p34^{cdc2}-like activity is significant in plant development. In the wheat leaf, cells that had recently left the meristem had residual p34^{cdc2}-like protein but did not activate it (Fig. 5.4) and here in the pea root cells could begin to accumulate p34^{cdc2}-like protein from early in germination but only activated it as it became appropriate to activate cell division in the tip meristem.

The distribution of cell division activity within the fully developed pea root has been established by Chaly and Setterfield (1975) who studied changes in cellular structure in the first 10 mm of pea root tip by excising 1-mm segments and revealed three main developmental zones in the cortex cells: 0-1 mm, cell division with compensating cell growth; 3-5 mm, rapid cell elongation; 7-10 mm, cell differentiation without further elongation. Distal to this region lateral roots develop and in 3-day-old pea root, lateral root primordia have formed in the region from 10-mm behind the tip to the base, although they have not emerged from the primary root, so cannot be seen with the naked eye (Wightman and Thimann, 1980). The formation of lateral root primordia is due to active division by one or two pericycle cells to form what at first appears to be multiple layers of pericycle cells. Soon a root apical meristem is organised from these and it grows outward through the cortex of the primary root as a lateral root. Although in primary root development only the tip meristem undergoes active cell division and cells leaving this region elongate and differentiate, it seems pericycle cells when differentiating keep the ability to rapidly re-enter the cell cycle and form initials for all cell types of the root.

The distribution of cell division activity in the developed root was consistent with a strong influence from p34^{cdc2} activation and with potential contribution from changes in the amount of p34^{cdc2}-like protein. The relative level of p34^{cdc2}-like protein to other proteins did not change dramatically but did maintained a correlation between higher level and active cell division. In the apical meristem (0-2 mm) it was slightly more abundant, and through the elongation and differentiation zone (2-10 mm) where division was at a minimum the level of the protein was a little lower than in the more distal regions, where lateral primordia were developing, and the level rose again slightly (Fig. 6.3 b). These data can be considered together with those derived from localisation of *Arabidopsis cdc2a* mRNA (Martines et al., 1992) or GUS activity driven by *Arabidopsis cdc2a* promoter (Hemerly et al., 1993), in *Arabidopsis* root. Messenger RNA and reporter gene activity levels indicated persisting presence of *cdc2* mRNA in the pericycle although in other cell types low levels occurred outside the apical meristem. The presence of persisting levels of p34^{cdc2}-like protein outside the meristem is consistent

with persistence of *cdc2* mRNA in the pericycle and supports the proposition of Hemerly et al. (1993) that presence of *cdc2* mRNA determines a potential for re-entering the cell cycle but the present data suggest that presence of p34^{cdc2} more directly fills this role.

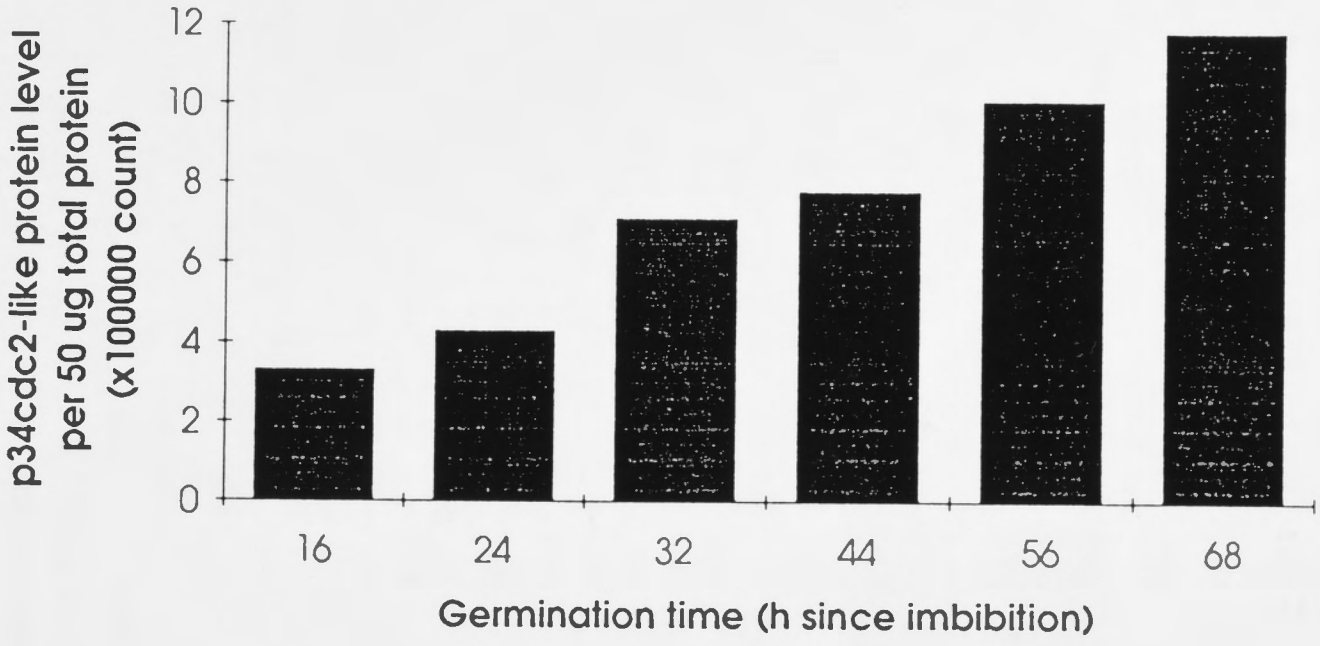
Controls acting on the level of p34^{cdc2} protein have been revealed by rapid change in p34^{cdc2} level in pea root in response to phytohormone level. Auxin stimulation of the elongation zone of pea root rapidly induces *cdc2* mRNA and p34^{cdc2}-like protein accumulation coincident with the stimulation of more abundant lateral root initiation, which suggests that the normally lower level could limit activity in this region (John et al., 1993b). In the same tissue, exogenous cytokinin applied after previous induction with auxin inhibits lateral root formation by causing breakdown of the induced p34^{cdc2} (John et al., 1993b). Thus the gently changing levels of p34^{cdc2} as cells progress through the developmental regions of the root (Fig. 6.3 b) result from the modulation of controls that can accelerate synthesis or breakdown of the key division protein.

The strongest potential determinant of division activity is the localised activity of p34^{cdc2}-like enzyme activity, which was sharply restricted to the terminal 0-2 mm of root (Fig. 6.4) in which the apical meristem lies (Chaly and Setterfield, 1975), although significant levels of p34^{cdc2}-like protein were present outside this region of most active division. Activity elsewhere in the root was only 25% of that in the tip region, comparing activities in equal amounts of extracted protein (Fig. 6.4 a). This comparison is probably the most meaningful since it gives an indication of the balance between the *cdc2* kinase and likely counteracting phosphatases and also an indication of the ratio of *cdc2* kinase to potential substrates. On a fresh weight basis p34^{cdc2}-like kinase activity declines even more outside the meristem, to 5%, but this decline is accentuated by a vacuolation of cells during differentiation that dilutes all proteins (Table 6.1).

The persistence of p34^{cdc2}-like kinase activity at low levels outside the tip meristem is in contrast with the developing wheat leaf (Fig. 5.3) and probably reflects the less homogenous presence of non-dividing cells in the root than in the leaf. Division is completely absent outside the wheat leaf meristem but in the root there is a less sharp demarcation with distance from the tip between regions of division and non-division. One reason for less sharp demarcation is the diversity of differentiated cell types which

requires cells that elongate more during differentiation such as vascular tissue to be formed from initials that cease division closer to the tip, whereas in leaf tissue vascular cells are at a lower ratio compared with the predominant mesophyll cells. The major reason for a less sharp transition to tissue containing non-dividing cells in the root is the requirement for some division to continue, unlike the determinate leaf. In the root continuing divisions are necessary to form the secondary vascular tissue and to initiate lateral roots, both of which occur within a few centimetres of the tip. The significant activity p34^{cdc2}-like kinase detected in tissues outside the tip meristem is entirely consistent with this continuing division activity.

Data from the diverse tissues of leaf and root therefore underline conclusions that have been drawn in our laboratory (John et al., 1993 a, b) and extend conclusions based on messenger RNA levels (Martinez et al., 1992; Hemerly et al., 1993) by confirming that levels of p34^{cdc2} protein are regulated in plant tissues and could be used to enforce absence of division. However it is also revealed that fine control of the extent of catalytic activation of p34^{cdc2}-like kinase is a crucial determinant and therefore demonstrates an urgent need in plants for the genetical and biochemcal basis of this control to be investigated and understood.



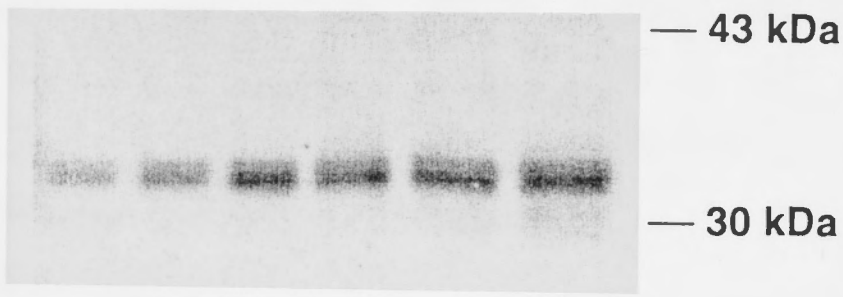


Fig. 6.1 p34*cdc2*-like protein level, relative to other proteins of the pea root, during the course of root development. Roots were excised at 16, 24, 32, 44, 56 and 68 h after imbibition (see Table 6.1). Samples containing 50 µg total protein were fractionated by electrophoresis and analysed by Western blotting with anti PSTAIR antibody (lower panel) and the PhosphorImage was analysed quantitatively to determine the amount of PSTAIR-containing protein of 34 kDa (upper panel). Material running a little more slowly (apparent M. W. 35 kDa) was included in the estimate since this is probably phosphorylated p34*cdc2* (Zhang et al., 1996).

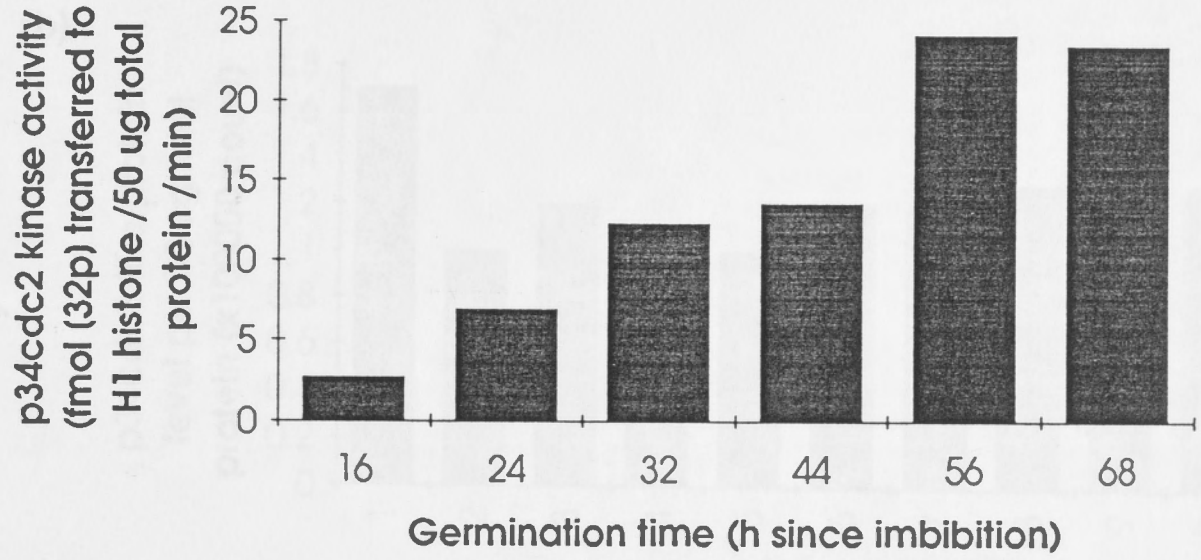
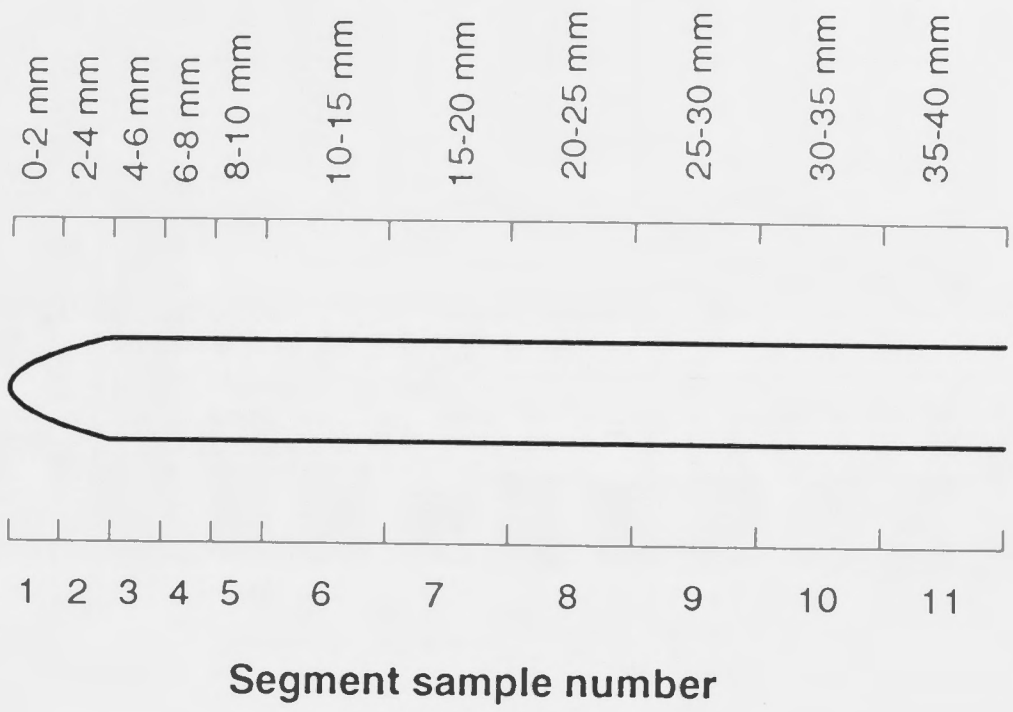
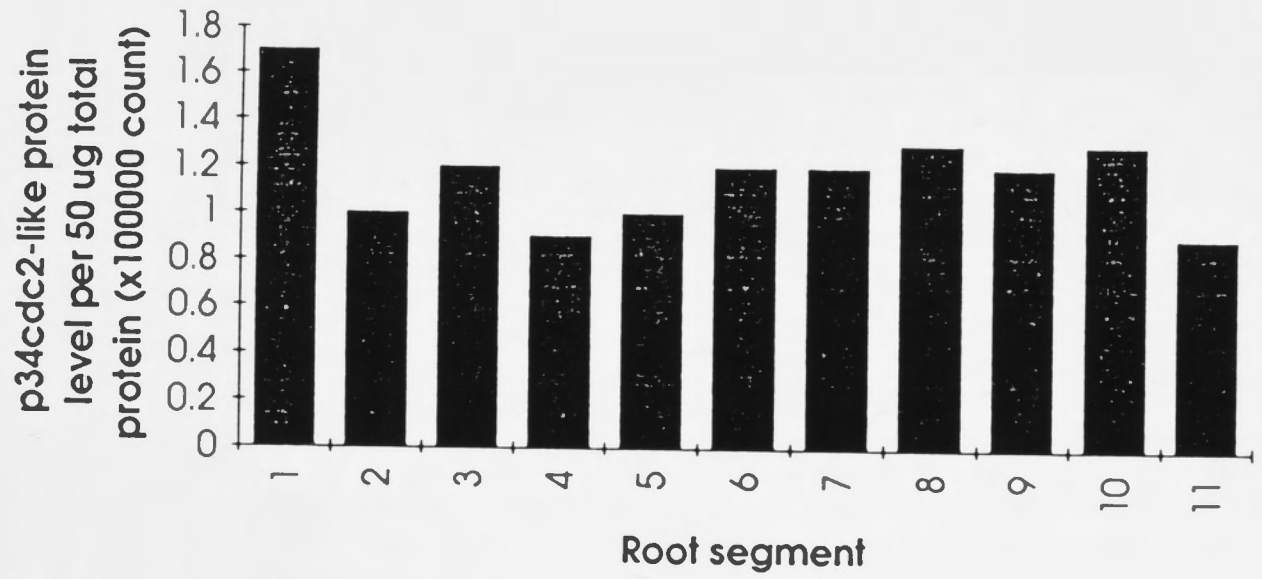


Fig. 6.2 p34^{cdc2}-like kinase activity of the pea root during the course of development. Enzyme was purified by p13^{suc1} beads before assay and activity was estimated as fmol [^{32}P] transferred to H1 histone per min per 50 μg total protein extracted from root tissue.

(a)



(b)



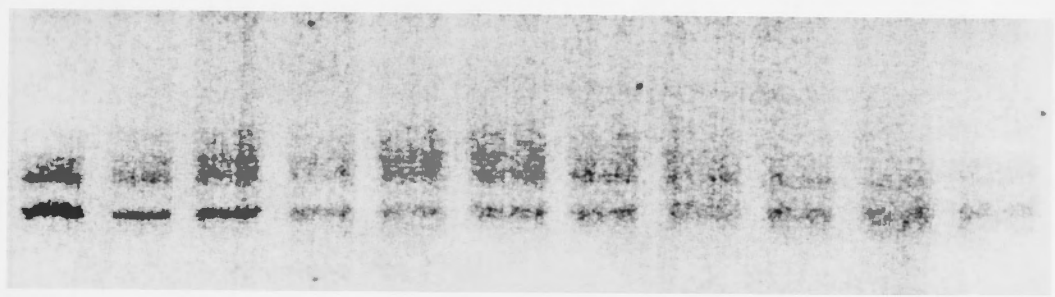


Fig. 6.3 p34^{cdc2}-like protein level in segments cut from 3-day-old pea roots. The segments were 2 mm long in the first 10 mm from the tip, and 5 mm long from 10 to 40 mm, as shown in (a), and their p34^{cdc2}-like protein level is shown in (b) which describes Western blotting of 50 μg extracted protein from each segment probed with PSTAIR antibody.

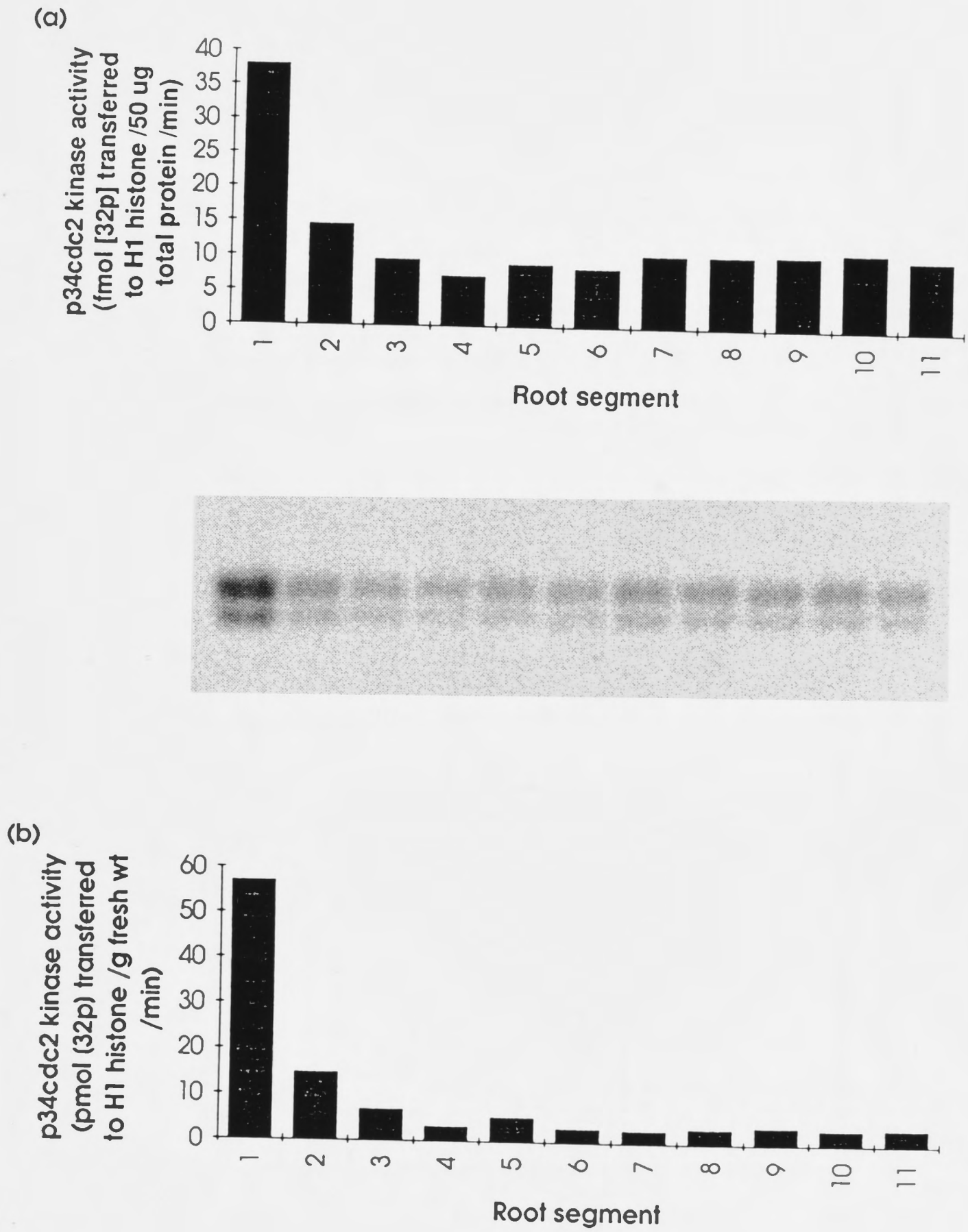


Fig. 6.4 p34*cdc2*-like kinase activity in segments taken from 3-day-old pea roots (as in Fig. 6.3). The enzyme was purified by p13*suc1* beads before assay and was estimated as fmol [^{32}P] transferred to H1 histone per min per 50 µg total protein extracted from root tissue as shown in (a); or as pmol [^{32}P] transferred to H1 histone per gram of fresh weight per min as shown in (b).

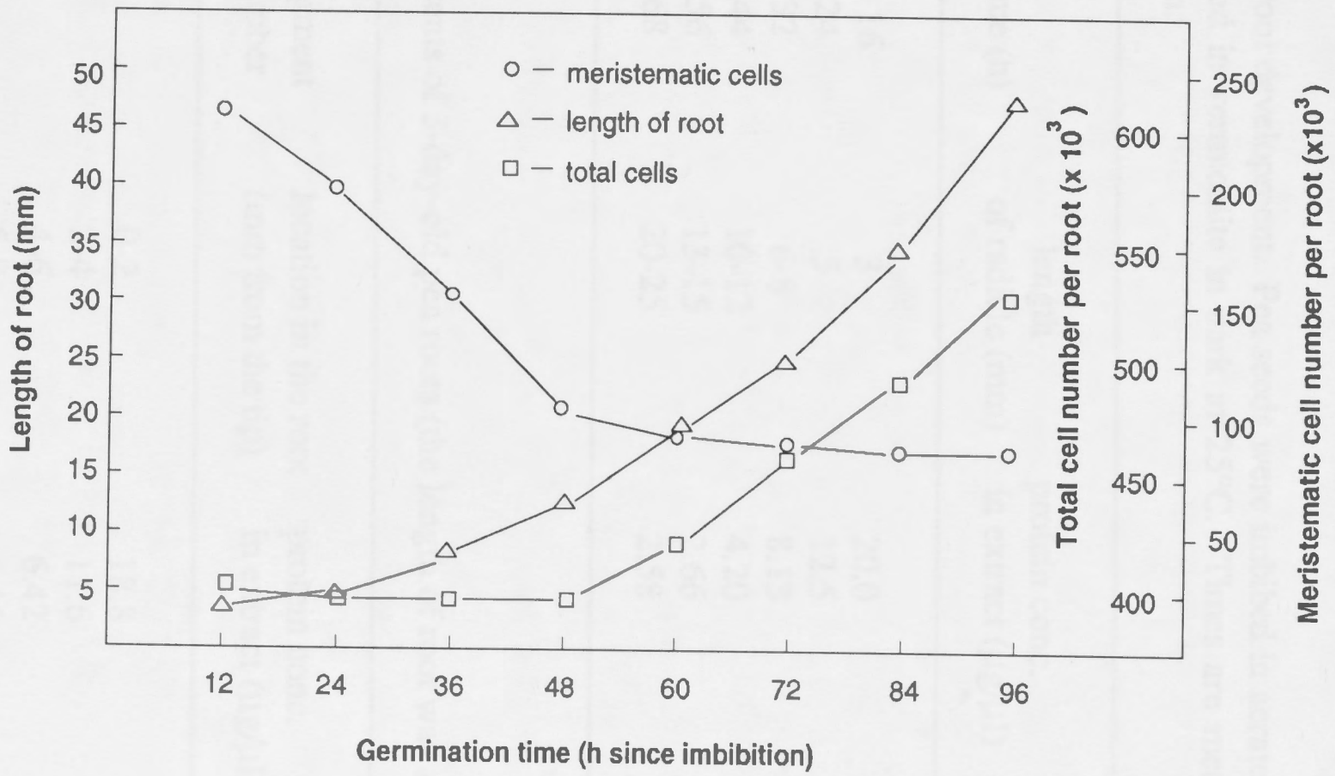


Fig. 6.5 Total length, total cell number and number of meristematic cells in root of different age. Data were provided by F. Wightman (unpublished data). Meristematic cells have been identified as small, isodiametric cells with dense cytoplasmic contents detected by chromic acid stain (as proposed by Brown, 1951). Total cell number was determined by haemacytometer counting of cells in suspensions obtained by macerating the pea roots in 5% chromic acid and repeated passage through the orifice of a pasteur pipette.

Table 6.1 Length of the emerging radicle and protein concentration (mg per g. f. w.), in radicle tissue during the first 68 h after imibition. Samples of pea root were frozen and ground in liquid nitrogen. Total protein was extracted from grindate by adding 300 µl NDE buffer (see Chapter 2, 2.2.5.2) to 0.1 g grindate and the protein concentration was determined by Coomassie dye binding assay (Chapter 2, 2.2.3.2). The same samples were also assayed for p34^{cdc2}-like protein level and kinase assay as described in sections 2.2.3 and 2.2.5, and results are shown in Figs 6.1-6.4.

(a) pea root development. Pea seeds were imbibed in aerated water for 8 h and then germinated in vermiculite in dark at 25°C. Times are measured from the start of imbibition.

time (h)	length of radicle (mm)	protein conc. in extract (µg/µl)	mean protein conc. in radicle (mg/g. f. w.)
16	3	20.0	80.0
24	5	12.5	50.0
32	6-8	8.13	32.5
44	10-12	4.20	16.8
56	13-15	2.66	10.6
68	20-25	2.58	10.3

(b) Segments of 3-day-old pea roots (the length of root was about 40 mm)

segment number	location in the root (mm from the tip)	protein conc. in extract (µg/µl)	mean protein conc. in segment (mg/g. f. w.)
1	0-2	18.8	75.2
2	2-4	11.6	46.4
3	4-6	6.42	25.7
4	6-8	4.46	17.8
5	8-10	4.07	16.3
6	10-15	3.15	12.6
7	15-20	3.53	14.1
8	20-25	3.01	12.0
9	25-30	3.70	14.8
10	30-35	3.44	13.8
11	35-40	3.51	14.0

Chapter 7
General Discussion

The present investigation of wheat *cdc2*-like genes, their mRNA level, protein level and enzyme activity during the development of leaf cells, together with the comparable study of *cdc2*-like protein involved in pea root development, has supported the hypothesis (John et al., 1990) that change in *cdc2* protein level participates in the control of plant development. No single experimental system in plants has previously allowed simultaneous study of mRNA, enzyme protein level and enzyme activity. The data suggest that the cessation of *cdc2* expression and a consequent low level of p34^{cdc2} protein may enforce the switch from division to differentiation, additionally the fine control of p34^{cdc2} kinase activity is identified as a crucial determinant for cell division. These conclusions are drawn both from the monocotyledonous wheat plant, in which the switch from cell division is essentially irreversible, and also from the initiation of meristem activity during development of a radicle in germinating pea.

To allow study of gene expression, wheat *cdc2*-like genes were cloned using PCR to amplify cDNA with primers derived from highly conserved regions of the *cdc2* gene (Chapter 3). Interestingly two *cdc2*-like cDNA fragments were amplified. Screening a wheat cDNA library with a PCR-fragment used as a probe also revealed these two *cdc2*-like cDNAs. They were 85% identical at the amino acid level and both contained characteristics of the *cdc2* gene, such as the "PSTAIR" domain, as well as common features of protein kinases such as the ATP-binding motif "GEGTYGVV" (Hanks et al., 1988). Both wheat genes have about 60% homology with the yeast *cdc2/CDC28* gene, 63% homology with the human *cdc2* gene and 82%-92% homology with other plant *cdc2*-like genes. Therefore these two genes were designated wheat *cdc2*-like genes *cdc2TaA* and *cdc2TaB*.

The *cdc2/CDC28* gene has been firmly established as a universal control element in the cell division cycle of eukaryotes (reviewed by Nurse 1990). In both fission and

budding yeasts, *cdc2/CDC28* plays a major role in controlling G1-S and G2-M transitions, but interestingly in animal cells *cdc2* appears to play a more restricted role being most important at G2-M phase, while the transition to S phase is governed by the product of a close variant of *cdc2* that has been termed the *cdk2* gene. Other CDKs with clearly divergent sequences have been recognised as filling roles during G1 phase and at the G0-G1 transition (see Chapter 1), therefore evolution in higher eukaryotes may have lead to a duplication and diversification of *cdc2* genes. The function of some CDKs in animal cells is not yet clear in spite of intensive study. Animal *cdk4*, *cdk5* and *cdk6* do not contain "PSTAIRE", instead they have "PV/ISTVRE", "PSSALRE" and "PLSTIRE" indicative of a generally lower sequence similarity with *cdc2* (40%-50%) (Meyerson et al., 1992; Matsushime et al., 1992; Tsai et al., 1994; Meyerson and Harlow, 1994). The situation in plant cells is even less clear although amplification from plant cDNA with PCR primers based on CDKs has recovered CDK-like gene sequences sufficiently different from *cdc2* to suggest that they might resemble the animal CDKs, that appear to be part of signal transduction pathways operating at G0-G1 rather than direct catalysts of the core cycle events of DNA replication and segregation (reviewed by Sherr 1994). Further evaluation of the more divergent plant CDKs will require cell cycle analysis to determine their time of expression and also biochemical analysis of their associated cyclins and favoured substrates, however it is intriguing that variants *cdc2c* and *cdc2d* of *Antirrhinum majus* have PPTALRE and PPTTLRE (Fobert et al., 1994); while *Arabidopsis thaliana cdc2b* also has PPTALRE (Hirayama et al., 1991). Even more remote is the pea *cdkPs3* which has 23 kDa carboxyl extension and the sequence PITAIRE (Jacobs 1995). Finally a rice protein R2 (Hata 1991) has 56% sequence identity with MO15 and could be the CDK-activating CDK.

In the present study I have concentrated on two genes that are more likely to be directly involved in the core cell cycle events of DNA replication and segregation, because of their close sequence similarity with *cdc2*. Because of the presence of *cdc2* together with *cdk2* in animal cells and their separate contributions to the core cycle events, the possibility that equivalent *cdc2* and *cdk2* genes are present in plants must be considered. Intriguingly almost all plants examined to date have two *cdc2*-like genes

with 80%-90% sequence similarity. They contain *cdc2* gene characteristic such as the PSTAIRE domain, and some of them can complement yeast $cdc2^{ts}$/$cdc28^{ts}$ mutants (discussed below). In animal cells, *cdc2* and *cdk2* are also relatively similar genes with 65% sequence identity, both contain the PSTAIRE domain and can bind to $p13^{suc1}$. It remains possible that the two *cdc2*-like genes found in several plant species may represent *cdc2* and *cdk2*.

One indication that supports this view is the different capacity of the two alfalfa *cdc2*-like genes to complement loss of *CDC28/cdc2* function in yeast, which could indicate that the alfalfa *cdc2MsA* gene has G2-M function characteristic of *cdc2* while *cdc2MsB* has G1-S function characteristic of *cdk2* (Hirt et al., 1993). The present study therefore tested yeast complementation by wheat *cdc2*-like genes and found that *cdc2TaA* could complement yeast $cdc28^{ts}$ mutants while *cdc2TaB* could not. This may indicate that the two wheat *cdc2*-like genes have different roles in the cell cycle, however caution is necessary in drawing this conclusion since complementation is not a decisive test of the identities of *cdc2* and *cdk2*. There is some evidence that *cdk2* identity can be diagnosed from inability to complement $cdc2^{ts}$/$cdc28^{ts}$ (reviewed by Pines and Hunter, 1991) since one situation in which complementation was detected but found to result from a second mutation in *CDC28* while the original $cdc28\text{-}4^{ts}$ was not complemented by *cdk2* (Elledge and Spottswood, 1991). This is consistent with a requirement for restoration of $cdc28^{ts}$ partial activity by secondary mutation before the remaining inactivity could be complemented by *cdk2*, which in animal cells has a more limited function than *cdc2/CDC28* has in yeast. However, there is evidence that some alleles of *cdc28* ($cdc28\text{-}13^{ts}$ and null-*cdc28*) can be complemented by *cdk2* with no additional mutation of the yeast enzyme (Ninomiya-Tsuji et al., 1991). Therefore the identity of *cdc2* or *cdk2* cannot simply be diagnosed by complementation. Sequence analysis of the non-complementing wheat *cdc2*-like gene *cdc2TaB*, seeking specific similarity with *cdk2* of human, frog and goldfish did not reveal any evidence that divergence of *cdc2TaB* from *cdc2TaA* introduced *cdk2*-like features. Of 23 conserved amino acids common to the *cdk2* gene but different to *cdc2*, none were found in equivalent positions in either *cdc2TaA* or *cdc2TaB*. Further more a comparison of the pair of wheat *cdc2*-like genes

and the pair of alfalfa *cdc2*-like genes also showed no evidence that regions of difference between wheat genes corresponded to regions of difference between the alfalfa genes, nor that the B-types showed any similarity at these points as they might if both performed Cdk2-like functions.

Therefore direct biochemical evidence will be required for the wheat *cdc2*-like proteins, indicating the time in the cell cycle at which they are enzymically active and the cyclins with which they associate, before it can be concluded whether any acts as a Cdk2-like molecule. If this is the case then plant Cdk2 molecules will differ in sequence from the animal equivalents.

One possibility is that plants are closer to yeasts than to animals in their requirements for executing START of division. In yeasts only low activity of p34^{cdc2} maybe required at START since repeated S phases follow inactivation of mutant cyclin B (*cdc13*^{ts}) (Hayles et al., 1994) and also low activity of H1 histone kinase is seen in synchronous cultures of *S. pombe* at time of entering into S phase (Hayles and Nurse, 1995). Study of p34^{cdc2} protein in fission yeast indicates that tyrosine phosphorylation and dephosphorylation of p34^{cdc2} are not required at initiation of S phase (Hayles and Nurse, 1995). In animal cells the necessity to restrict cell proliferation to appropriate circumstances, although bathing fluids contain many biosynthetic precursors that could support division, involves species of cyclin and remote variants of Cdc2 that have no parallel in yeasts but allow control of proliferation by many hormonal, anchorage and contact inhibition cues. It is conceivable that multicellular plants more closely resemble the yeasts than higher animals. Plant cells are clearly not bathed in rich nutrient fluid and hormonal mechanisms partially control the distribution of nutrients (Luttge and Higinbotham, 1979). Plants usually retain meristematic regions of active cell division and continue to enlarge throughout their life in a way that animals do not (Chapter 1, 1.8). Although there is liquid flow through a plant there is not the rapid circulation of the animal vascular system. The dual transport system of plants, comprising xylem and phloem, is specialised to carry different classes of molecule usually unidirectionally, with the result that only the meristems receive an on-going supply of all growth precursors (Flowers and Yeo, 1992). Thus growth in the plant is influenced by structural and

hormonal factors governing nutrient supply that are not available as controls in animals. Additionally plant hormones are small molecules not peptides, therefore the signal transduction pathway in plants need not involve membrane receptors and second messengers to the extent that they occur in animal cells, although this area is currently little understood in plants. It is entirely possible therefore that hormonal signals in plants are not coupled to the network of CDK proteins that are active in G1 phase in animal cells (reviewed by Sherr 1994). There is some direct evidence for different connections between hormones and the cell cycle in plants in that the cytokinin class of plant hormone, which by ratio of concentration to auxin hormones governs the initiation lateral meristems and hence branching of plants (Torrey 1956), acts at the G2-M transition in controlling plant cell proliferation (Zhang et al., 1996). In this respect plant cells differ from mammalian, which cease to require hormonal stimulation after passing late G1 phase (Pardee 1978; Zetterberg and Larsson, 1985), however plant cells use tyrosine phosphorylation of Cdc2 as the biochemical mechanism of their cell cycle control at the G2-M transition as do yeast cells (Zhang et al., 1996). Another point of difference is that plant cells that are arrested in cycle progress by lack of cytokinin do not enter apoptosis as non-malignant hormone-deprived animal cells do. The plant cells can remain for more than a week in suspended cell cycle progress and when given cytokinin resume by dephosphorylating p34^{cdc2} and entering mitosis (Zhang et al., 1996).

Given these differences between plant and animal cell cycle control it will be fascinating to explore the possibility that wheat Cdc2TaB may be specialised to perform a late G1 (Cdk2-like) function although lacking structural affinities that are specific for animal Cdk2. It may, like yeast Cdc2 in late G1, become active on binding with an appropriate cyclin without involving change of tyrosine phosphorylation. The parallel with fission yeast suggests that it may be of lower abundance or lower catalytic activity than the mitotically active enzyme (Hayles et al., 1994).

Despite the uncertainty concerning the precise cell cycle function of *cdc2TaB*, complementation by *cdc2TaA* supported cell division in yeast mutants *cdc28-13*^{ts} and *cdc28-1N*^{ts} (Chapter 4), therefore the *cdc2TaA* gene is a functional homologue of *cdc2/CDC28*. Its cloning provided two reagents that could be used to analyse plant

development. One was the gene itself, which allowed probing for the *cdc2* mRNA. Another was antibody raised against the carboxy-terminal sequence of the protein deduced from the gene, which offered greater specificity for *cdc2* protein than antibody directed against the PSTAIRE sequence that has been used hitherto in studies of wheat. The PSTAIRE antibody is potentially less specific for Cdc2 since it could also detect CDKs that retain the 16 amino acid PSTAIRE sequence (Meyerson et al., 1992).

Using these reagents, wheat *cdc2*-like gene expression and *cdc2*-like protein level was found to correlate with high division activity in the leaf meristem region, while in mature leaf tissue *cdc2*-like mRNA was hardly detectable (Fig. 3.13). The $p34^{cdc2}$ protein level relative to other proteins was high in the meristem and declined as cells switched to differentiation. In mature leaf tissue the $p34^{cdc2}$ protein level declined to a basal level of about one fifteenth of the highest level detected in meristem (Fig. 5.4). This finding indicates that the cessation of *cdc2* gene expression may cause the development of a low level of $p34^{cdc2}$ protein that in turn enforces cessation of division and the switch to cell differentiation.

Changes of *cdc2* mRNA level and $p34^{cdc2}$ protein level have been found to correlate with developmental stages in animal and plant cells. Quiescent human cells have very low levels of *cdc2* mRNA and protein but on re-entering the cycle the amount of *cdc2* mRNA and protein increase (Lee et al., 1988; Welch and Wang, 1992). In chicken and *Drosophila*, there is a positive correlation between the proliferative state of tissues and the abundance of *cdc2* mRNA (Krek and Nigg, 1989; Lehner and O'Farrell, 1990a). In plants, evidence from alfalfa (Hirt et al., 1993; Magyar et al., 1993), maize (Colasanti et al., 1991), rice (Hashimoto et al., 1992), petunia (Bergounioux et al., 1992), soybean (Miao et al., 1993) and *Arabidopsis* (Martines et al., 1992; Hemerly et al., 1993) also show that the high level of *cdc2* mRNA positively correlates with the proliferative state of the organs. It has therefore been proposed that change in *cdc2* expression participates in control of cell development. However, the earlier studies of plants, either investigated only mRNA level or only protein level. A novel feature of the present study is that in a single plant species *cdc2* mRNA and *cdc2* protein level were compared and revealed that cessation of *cdc2* gene expression precedes the decline of *cdc2* protein. So the low level

of p34^{cdc2} protein seen outside the meristem could most simply result from the cessation of *cdc2* transcription together with the dilution of existing *cdc2* protein by other proteins whose accumulation continues during cellular differentiation.

The significance of decline in *cdc2* protein level relative to other proteins during development was underlined by the study of root development during pea germination (Chapter 6), which offered the possibility of testing a positive association between *cdc2* and plant division and revealed that initiation of cell division in pea root does require an increase in level of p34^{cdc2}-like protein relative to others. This observation correlates with an earlier observation (Gorst et al., 1991) that resumption of division in excised carrot cotyledon tissue required restoration of the relative level of p34^{cdc2}-like protein to that found during the earlier cell proliferation phase. Both these observations bear upon the finding that when the amount of p34^{cdc2}-like protein was calculated per cell in wheat leaf tissue during the transition from division to differentiation the amount of *cdc2* protein per cell was found not to decline although it was diluted fifteen fold by other proteins (John et al., 1990). If the protein was localised to a specific region of the cell this dilution might not have been significant. The present data from pea and the earlier data from carrot (Gorst et al., 1991) suggest that low p34^{cdc2} is significant since the level of Cdc2 protein relative to others must be restored prior to division. It can be speculated that the biological significance of the relative levels of Cdc2 protein to other proteins is that synthesis of proteins other than Cdc2 decreases the frequency with which Cdc2 encounters its substrates and also decreases the capacity of Cdc2 to maintain phosphorylate its substrates due to the increasing tendency of phosphatases to counter act the activity of the Cdc2 kinase.

Considering the possible advantages of lowering Cdc2 protein level rather than holding the protein in an enzymically inactive state, it can be noted that inactivation of Cdc2 enzyme may be metabolically more expensive or, in the long term, insufficiently secure. Inhibition of p34^{cdc2} by phosphorylation of tyrosine could incur both of these difficulties because it has been observed that phosphatases not directly involved in the cell cycle can remove the inhibitory phosphate (Gould et al., 1990) therefore inhibition may be difficult to maintain indefinitely and could require continuing re-phosphorylation.

Additionally removal of the cyclin subunits that p34^{cdc2} requires for activity may also be difficult because it has recently been detected that protein kinases not involved in the cell cycle can have associated cyclins (Espinoza et al., 1994) and considerable non-specificity of cyclin action has been revealed by the finding that all known classes of cyclin can complement lack of the yeast G1 (CLN1-3) cyclins (reviewed by Sherr 1993) and further revealed by the capacity of mitotic cyclin A to advance the initiation of S phase (Resnitzky et al., 1995). Furthermore, presence of p34^{cdc2} without functional cyclin B does not lead to cycle arrest but to abnormal cycle progress involving multiple repetition of S phase (Hayles et al., 1994). There are therefore many likely reasons and it is clear that reduced level of Cdc2 protein is widely used in plants to enforce the cessation of division in cells that are enlarging as part of their differentiation and would otherwise be eligible to divide.

Although plants use reduced level of p34^{cdc2} as a long term control in differentiation, the enzyme is under fine control of activity, as can be seen from the peak of H1 histone kinase activity that accompanies mitosis in synchronous culture (John et al., 1993a) while the level of p34^{cdc2} protein is unchanged through the cell cycle (Zhang et al., 1996). The biochemical basis of this change in activity at mitosis is the inhibitory phosphorylation of tyrosine in p34^{cdc2} since the enzyme from plant cells that are in late G2 phase has high levels of this phosphate and can be activated *in vitro* by Cdc25 phosphatase that is specific for removal of phospho tyrosine from the ATP binding region of Cdc2 (Zhang et al., 1996). It is entirely consistent with the operation of such fine control of enzyme activity in the Cdc2 protein that cell division in plant tissue correlates more precisely with catalytic activation of p34^{cdc2}-like protein kinase than with simple presence of the protein. In wheat seedling leaf high activity of p34^{cdc2}-like protein kinase was restricted to the region where active cell division occurred (Fig. 5.1 and 5.3) and in differentiating or mature cells the activity of p34^{cdc2}-like kinase was very low although the cells beginning differentiation contained appreciable p34^{cdc2} protein (Fig. 5.4). Furthermore, during pea root development, activation of p34^{cdc2}-like kinase was most rapid from 44 h to 56 h when cells began dividing and the activity of p34^{cdc2}-like kinase reached a plateau at 56 h (Fig. 6.2 and 6.5). In fully developed pea

root, high activity of p34^{cdc2}-like kinase was confined in the root tip (Fig. 6.4) in close parallel with the seedling leaf.

The activation of p34^{cdc2}-like protein in association with division activity in wheat leaf and pea root meristems probably involves association with cyclin to form active holoenzyme and also changes in phosphorylation under the control of regulators such as Cdc25, CAK, Wee1, Nim1 (reviewed by Nurse 1990; King et al., 1994; also see Chapter 1). In *Arabidopsis*, expression of a cyclin gene (*cyc1At*) is found to be almost exclusively confined to dividing cells (Ferreira et al., 1994 b); in a cyclin transgenic *Arabidopsis*, GUS activity induced by *cyc1At* promoter is found to be high in primary root apices and lateral root primordia (Ferreira et al., 1994 b). So cyclin expression is one candidate for a spatial control of p34^{cdc2} kinase activity during organogenesis, however this may be a secondary control since mitotic cyclin synthesis is part of an on-going cell cycle (reviewed by Nasmyth 1993) therefore the primary initiator of localised divisions may remain to be discovered. It would be interesting to follow the present study with an investigation of G1 cyclin expression in wheat and pea, since this class of cyclin could be an initiator of the cell cycle.

The present study may have revealed part of the molecular basis of the recalcitrance of monocotyledonous cells in re-entering the cell cycle. In contrast with the response of dicotyledonous tissues (Gorst et al., 1991; Zhang et al., 1996) auxin treatment did not elevate p34^{cdc2} level in wheat leaf segments (Chapter 5). Auxin used in this study was at the concentration and under conditions found to be most effective for inducing cell division and callus formation in wheat (Wernicke et al., 1986). Combinations of auxin with cytokinin at different concentrations were also tested (data not shown), because in some species this combination is more effective in inducing division in tissue culture than auxin or cytokinin alone, as found in tobacco tissue culture (Simard 1971). However treatment with combinations of auxin and cytokinin was less effective in obtaining cell proliferation in wheat than auxin alone. The irreversible loss of ability to resume division in cereals is a common phenomenon (reviewed by Vasil 1994) and may correlate with the absence of any activation of secondary meristems, either during normal ontogenesis or under stress conditions as for instance after wounding. The difficulty of inducing cell

division currently limits the application of gene transfer technology in cereals. The present study, in showing the difference of *cdc2*-like gene expression in response to auxin treatment between cereals and dicotyledonous plants, provides a likely explanation of their different capacity for resumption of division and suggests that it could be profitable to explore the control of cdc gene expression as a means of allowing genetic manipulation in cereals.

Presence of multiple *cdc2*-like genes in plants could indicate that their promoters respond to different hormones. In deepwater rice, gibberellin (GA) can promote rapid internodal growth by increasing the transcriptional levels of one *cdc2*-like gene (*cdc2Os2*) and cyclin genes (*cycOs1* and *cycOs2*) (Sauter et al., 1995). Since wheat and rice are closely related and the pair of wheat *cdc2*-like genes are most like the pair of rice *cdc2*-like genes (Chapter 3), it would be interesting to explore the possibility that the wheat *cdc2*-like genes may be used under different physiological conditions. The alternative possibility (discussed earlier), that they have specific functions with in the cell cycle could be explored by developing well synchronised cell cultures and specific antibodies to particular *cdc2*-like genes and to plant G1 cyclins or G2 cyclins to determine what CDKs are in complex and active in particular cell cycle phases.

To further study the regulation of *cdc2*-like genes during the plant development, promoter isolation and analysis would provide information concerning *cdc2* gene response to internal developmental signals and environmental signals such as drought and salinity. The further investigation of cyclin genes and other regulators such as Cdc25, CAK and CKIs in plants will greatly enrich our understanding of cell cycle control as well as the relationship between cell cycle control and cell development in plant kingdom.

APPENDIX

Media Used in This Study

1. MS medium

	mg/liter	mM
NH_4NO_3	1650	20.6
KNO_3	1900	18.8
$CaCl_2 \cdot 2H_2O$	440	3.0
$MgSO_4 \cdot 7H_2O$	370	1.5
KH_2PO_4	170	1.25
		µM
KI	0.83	5.0
H_3BO_3	6.2	100
$MnSO_4 \cdot 4H_2O$	22.3	100
$ZnSO_4 \cdot 7H_2O$	8.6	30
$Na_2MoO_4 \cdot 2H_2O$	0.25	1.0
$CuSO_4 \cdot 5H_2O$	0.025	0.1
$CoCl_2 \cdot 6H_2O$	0.025	0.1
Na_2EDTA	37.3	100
$FeSO_4 \cdot 7H_2O$	27.8	100
sucrose	30 g/liter	
pH	5.7	
Solid MS medium	8 g Bacto-Agar/liter	

2. LB medium

	g/ liter
Bacto-tryptone	10
Bacto-yeast extract	5
NaCl	10
pH	7.0
(agar	15)

3. SOB medium

	g/liter
Bacto-tryptone	20
Bacto-yeast extract	5
NaCl	0.5
KCl	0.186
pH	7.0

4. Terrific Broth

To 900 ml of deionized H_2O, add

Bacto-tryptone	12 g
Bacto-yeast extract	24 g
glycerol	4 ml

After autoclaving and the solution cooling down, add 100 ml of
0.17 M KH_2PO_4, 0.72 M K_2HPO_4 sterile solution.

5. YEPD medium

	g/liter
Bacto-yeast extract	10
Bacto-peptone	20
glucose	20
adenine	0.01
uracil	0.01
(agar	20)

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